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Biotechnological Approach to a new Foot-and-Mouth Disease Virus Vaccine

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Introduction

Advances in biotechnology in the past decade have opened up new ways of manufacturing specific proteins with micro-organisms. This novel approach is the result of several major observations and discoveries.

Cohen, Chang and Hsu (1972) observed that bacterial cells can be transformed or transfected with plasmid or viral deoxyribose nucleic acid (DNA), allowing the isolation of individual DNA sequences. Smith and Wilcox (1970) and Kelly and Smith (1970) discovered Class II restriction enzymes that recognize specific nucleotide sequences and cleave phosphodiester bonds within the recognition sites, permitting the purification of defined segments of a genome. With the use of ligase, chimeric DNA molecules can be created *in vitro* from DNAs of diverse origins and replicated in a bacterial host after transformation. Maxam and Gilbert (1977) and Sanger, Nicklen and Coulson (1977) developed rapid methods for determining the nucleotide sequence of DNA molecules, thus enabling the deciphering of genetic information encoded by the cloned DNA molecules. In recent years, the degree of sophistication and the range of application of this technology has increased considerably; it is now a routine procedure for studying the structure and organization of individual genes. Once a desired gene is identified, it can be manipulated to be expressed in an appropriate host, where the gene product can be produced in large quantities. With the progress of recombinant DNA technology, a revolutionary concept of vaccine production has been envisaged.

Conventional vaccines comprise (1) specific compounds purified from the pathogenic agent, (2) the whole killed pathogenic agent, or (3) an avirulent strain of the pathogenic agent as a live vaccine. There are intrinsic problems associated with conventional vaccines. For killed vaccines, complete inactivation of the pathogenic organisms is of the greatest concern, because application of an inadequately inactivated vaccine will lead to infection with, instead of immunization against, the disease. With attenuated live organisms there is the danger

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that they may mutate and revert to the virulent form and so infect the recipient that they are intended to protect.

The use of genetic engineering technology to manufacture vaccines has several potential advantages over conventional production methods. For all practical purposes, genetically engineered vaccines are safe because they are free from the causative agent. There is no fear of inadequate inactivation or reversion to the virulent form, since only parts of the genome of the pathogenic organism are cloned and used for vaccine production; the antigens are produced in the absence of the whole infectious agent. There is no risk of generating infectious DNA, RNA or virion. It is possible to manipulate bacterial cells to express a recombinant polypeptide at levels up to 20–30% of the total cellular proteins. Bacterial cultures require less space, time, cost and effort to maintain than tissue cultures, embryonic eggs or experimental animals; therefore, it is more cost effective to produce specific antigens in bacteria. Hence, even at the expense of low yield in certain purification schemes to eliminate contaminants, one can well afford to increase the purity of the preparation. In cases where only limited quantities of certain polypeptides are synthesized in their natural environment, production by recombinant DNA technology is definitely a more practical approach.

Unfortunately, it appears that foreign polypeptides synthesized in bacterial hosts in some instances do not possess the same antigenic activity as that normally displayed by the 'natural' polypeptides and, therefore, are less effective antigens. This may be because cloned polypeptides formed in a different environment, i.e. the bacterial rather than the normal host cell, may not be folded in the same way to generate the same active sites or antigenic determinants as those that are present on the native polypeptides. Consequently, these cloned proteins will not perform the desired functions or will exhibit reduced activities. It is also possible that contamination with materials of bacterial origin, not separable from the cloned product, might cause undesirable side-effects.

Current control programmes against FMD

Foot-and-mouth disease (FMD) is one of the most feared and devastating diseases of animals because it is highly contagious. Transmission of the FMD virus (FMDV) has at various times been attributed to wild and domesticated animals, animal products, human beings, rodents, birds, insects and the wind (Westergaard, 1982). The virus infects cloven-hoofed animals which include economically important livestock such as cattle, swine, sheep and goats. The disease is characterized by vesicular lesions on the feet and the oral area of the infected animal; other parts of the body e.g. snout, teats, skin, rumen and myocardium, may also be involved. Although the disease, in general, only lowers the productivity of the adult animals because of poor body condition, mortality among young animals can be as high as 50%. In practice, infected animals are slaughtered and their carcasses disposed of safely. The damaging effects of an epizootic are considerable: not only is there a direct loss of meat, dairy products,

wool, hides and other animal products; there is also an extremely costly indirect loss due to the shut-down of international markets.

A few countries (e.g. Australia, New Zealand, the United States, Canada and Japan) have been free of FMD for long periods. The last outbreaks in the United States and Australia were in 1929 and 1872, respectively. Denmark was free of FMD from 1970 until an outbreak occurred in March 1982 on the islands of Funen and Zeeland. These countries, and others such as the UK, do not immunize their livestock against FMDV. If an outbreak occurs, the disease is eradicated by slaughtering the infected and exposed animals and disposing of the carcasses. The major factor contributing to the fact that these countries remain FMD free is probably due to their effective policies in restricting importation of animals and animal products from countries where FMD is a continuing problem.

Most European countries started vaccination programmes in the early 1950s and have continued their efforts systematically. These countries have been able to keep FMD to low levels, and outbreaks occur only sporadically. At present, three categories of vaccination policies are practised in Europe: (1) no vaccination in the northern and north-eastern regions; (2) vaccination programmes in border areas of eastern countries; and (3) regular prophylactic immunization in western and central regions.

The principal reasons for not vaccinating the animals are: (1) susceptible herds are the best indicators for the presence of FMDV; (2) eliminating the risk of generating carriers for the disease through vaccination of the animals. However, when an outbreak occurs, the situation can be devastating in regions where vaccination is not practised. During the 1967–68 epizootic in England, approximately half a million head of infected or exposed cattle, swine, sheep and goats were slaughtered.

In South America, even though there has been a long practice of vaccination programmes, FMD has not been reduced to an acceptable level. In 1981, more than 500 million doses of vaccines were produced for South America, which makes this part of the world the largest market for FMDV vaccine. It is encouraging that the FMD incidence has declined significantly in recent years since the introduction of strict quality control for the vaccines. In Asian and African countries with strict vaccination programmes, the disease has been brought to low levels, whereas in countries with limited vaccination programmes, FMD continues to be endemic.

Current FMDV vaccines

At present, live attenuated and inactivated whole-virus vaccines are used for the control of FMD, with the inactivated vaccine being the most commonly used. These vaccines are multivalent and they include combinations of the seven serotypes prevalent in certain regions of the world. In Europe and South America, trivalent or quadrivalent vaccines which are composed of chemically inactivated viruses of serotypes A, O and C are used. Varying proportions of the virus types are incorporated into the vaccines. The composition of the vaccine depends on the immunogenicity of the viral particles used and the response in

target animals, because some serotypes are better immunogens than others and because some animals respond better than others. It has been reported that larger amounts of serotype O viruses than serotype A or C viruses are required to protect swine, indicating that serotype O virus is intrinsically a poorer immunogen (McKercher and Farris, 1967; Bachrach and McKercher, 1972). The vaccine dosage for different animals also varies, and more viral antigen is needed to immunize swine than cattle (Morgan, McKercher and Bachrach, 1970). In some cases, subtype-specific vaccines are necessary because some subtype viruses within the same serotype differ significantly in their immunogenicity and give unsatisfactory levels of cross-protection. Duration of immunity induced by two inoculations of whole-virus vaccine lasts about one year in cattle but not as long in swine. In Europe, where FMD is sporadic, animals are vaccinated once or twice a year; in South America, where the disease is frequent, animals are vaccinated two or three times a year.

LIVE ATTENUATED VACCINES

The general practice for generation of avirulent viral strains has been serial passage of virulent strains in an 'unnatural' host, e.g. in tissue cultures or in animals other than the usual host. Such procedures have been very successful in generating attenuated strains for several animal diseases including canine distemper, rinderpest, equine influenza and vesicular stomatitis. There had been considerable effort to obtain avirulent strains of FMDV (Skinner, 1959, 1960; Mowat, Barr and Bennett, 1969; Dietzschold, Kaaden and Ahl, 1972). However, those strains that elicit a good immune response generally give low but significant levels of lesions and side-effects; those that do not provoke lesions elicit poor immunity in animals. There is also the danger that an avirulent strain may revert to its virulent form, and that the vaccine thus may cause a serious epizootic instead of preventing the disease. As a result, modified live-virus vaccines have not been widely used.

INACTIVATED WHOLE-VIRUS VACCINES

Chemically inactivated virus is the most commonly used vaccine against FMD at present. The viruses are produced in suspension or monolayer cultures of baby hamster kidney cells (BHK-21, clone 13) (Mowat and Chapman, 1962; Capstick *et al.*, 1965) or bovine tongue epithelial explants (Frenkel, 1951). Chemicals that have been used to inactivate the harvested virus include formalin, glutaraldehyde, β-propriolacton (BPL) or aziridine derivatives such as acetylethylenimine (AEI) or binary ethylenimine (BEI) (Brown *et al.*, 1963; Brown, Cartwright and Stewart, 1963; Sangar *et al.*, 1973; Bahnemann *et al.*, 1974; Bahnemann, 1975).

There has been a gradual decrease in the use of formalin and an increase in the use of AEI or BEI as the inactivating agent (Olascoaga *et al.*, 1982) in vaccine production, because formalin inactivation does not follow first-order kinetics and the aziridine derivatives are safe and easy to handle. Recent outbreaks (1979, 1981) in France (Brittany and Normandy) and in the United

Kingdom (Jersey and the Isle of Wight) have been associated with the incomplete inactivation of FMDV with formalin and subsequent virus spread from areas where vaccination was practised (King *et al.*, 1981). New methods of virus inactivation are still being explored and preliminary results for virus inactivation through the activation of virion-associated endonuclease at alkaline pH in the presence of monovalent ions are promising (Scodeller *et al.*, 1982).

There are several disadvantages associated with the production and use of killed vaccines. These include the handling of large volumes of infected cultures; a requirement for biological containment facilities; a requirement for refrigeration during storage and distribution, especially in tropical countries, because of the heat-labile nature of the viral particle; and the poor growth of some subtypes in culture to yield adequate amounts of antigen. Nevertheless, the present chemically killed virus vaccine is quite safe and effective, provided that complete inactivation is achieved.

General structure and molecular organization of FMDV

FMDV is an aphthovirus of the family Picornaviridae; other genera of the family include enteroviruses, rhinoviruses and cardioviruses. The genetic information of FMDV is encoded in a single-stranded RNA of molecular weight $2{\cdot}8 \times 10^6$ daltons, which corresponds to approximately 8000 nucleotides (*Figure 1a*). The viral RNA is infectious and can serve as messenger RNA (mRNA). The viral RNA is not capped; instead, a small viral-specific polypeptide (VPg) of molecular weight 4000 daltons is covalently linked to the uridylic acid at the 5′ terminus of the RNA via a tyrosine residue (Sangar *et al.*, 1977; Grubman, 1980; Forss and Schaller, 1982). A polycytidylic acid tract, poly (C), of 100–170 bases (Brown *et al.*, 1974) is located approximately 400–500 bases from the 5′ end of the RNA molecule (Harris and Brown, 1976; Rowlands, Harris and Brown, 1978). The major protein synthesis initiation site is present on the 3′ side of the poly (C) tract (Harris, 1979; Sangar *et al.*, 1980). A single polyprotein translation product is produced from the RNA and is subsequently cleaved. The intermediate cleavage products are then further processed to give the mature non-capsid and capsid viral proteins (VP) by host-specific and viral-specific proteases (Sangar *et al.*, 1977, 1980; for review *see* Sangar, 1979). In common with most eukaryotic mRNAs, the viral RNA carries a poly (A) tail at its 3′ terminus.

As in all other picornaviruses, the protein shell is composed of four major polypeptides (VP1, 2, 3 and 4). The viral particle is icosahedral, 22 nm in diameter, and consists of 60 copies of each structural protein and one or two copies of VP0, the uncleaved precursor of VP2 and VP4 (Vande Woude, Swaney and Bachrach, 1972). VP1, 2 and 3 have molecular weights of around 30 kilodaltons (kd) and VP4 is approximately 13·5 kd, as estimated by polyacrylamide gel electrophoresis (PAGE) (Burroughs *et al.*, 1971). All four are phosphorylated (La Torre *et al.*, 1980). The native virion sediments at 146S. By lowering the pH to 6·5 or heating at 56°C, the virion dissociates into 12S particles which consist of five copies each of VP1, VP2 and VP3, and an aggregate of VP4 molecules (Burroughs *et al.*, 1971; Vasquez *et al.*, 1979).

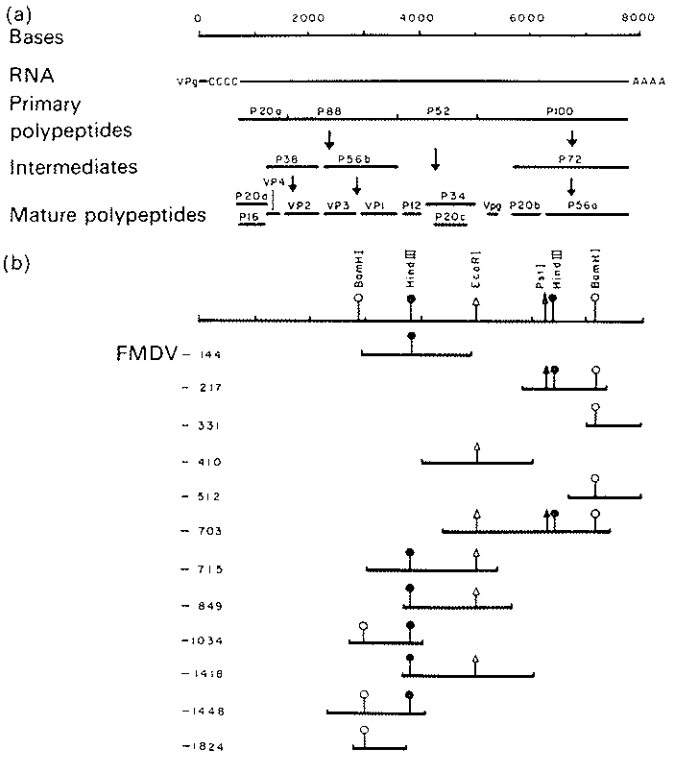


Figure 1(a) Biochemical map of foot-and-mouth disease virus. (Based on data of Sangar *et al.*, 1977 and Sangar, 1979).
(b) Restriction map and cDNA clones of FMDV strain O_1K. (*See* text for details. Courtesy of Küpper *et al.*, 1981).

Considerable confusion has been caused by the behaviour of the major capsid proteins of FMDV in various electrophoretic resolution systems. VP1, VP2 and VP3 in SDS-PAGE (Rowlands, Sangar and Brown, 1971) correspond to VP3, VP2 and VP1, respectively, as defined by urea-containing gel (Matheka and Bachrach, 1975). To resolve this confusion, each polypeptide has been named after its *N*-terminal amino acid residue, which is the same in all viral strains so far analysed, i.e. VP_{Thr}, VP_{Asp} and VP_{Gly} (Strohmaier, Wittmann-Liebold and Geissler, 1978) or VP_T, VP_D and VP_G (Bachrach, Morgan and Moore, 1979) which correspond to VP1, VP2 and VP3 in SDS-PAGE, respectively. The most recent designation is in favour of the SDS-PAGE terminology (American Society of Virology Meeting at Ithaca, New York, 1982), in conformity with the picornaviridae nomenclature. According to this system, VP1 of FMDV corresponds to VP1 of poliovirus and α of cardioviruses. In this review we have adopted the latest nomenclature.

At present, different strains of FMDV are categorized by serological assays such as complement fixation and neutralization tests. Seven serotypes, namely European (A, O and C), South African Territories (SAT1, SAT2 and SAT3) and Asian (Asia1) are recognized. Antibodies elicited by a particular serotype do

not neutralize viruses of a different serotype; consequently, animals immunized with a particular FMDV serotype are not protected from infection by any of the other six heterotypic viruses. In addition, variation within each serotype has resulted in over 65 known subtypes. Reduction in cross-reactivity among subtypes of the same serotype can be quite significant. Animals immunized against one subtype may not be protected against infection by a different subtype.

Identification of the immunizing viral protein—VP1

REDUCTION OF ACTIVITIES OF PROTEASE-TREATED VIRUS

When serotype O viruses are incubated with trypsin (Wild and Brown, 1967; Rowlands, Sangar and Brown, 1971; Strohmaier and Adam, 1974; Strohmaier, Franze and Adam, 1982) or upon long incubation with chymotrypsin (Cavanagh *et al.*, 1977; Barteling *et al.*, 1979), the ability of viral particles to adsorb to host cells is greatly decreased and infectivity is reduced 100–1000-fold. The ability of the protease-treated virus to elicit neutralizing antibodies is also decreased 100–1000-fold. It was concluded that the cell attachment site and immunizing properties of FMDV serotype O viruses are closely associated with this enzyme-sensitive peptide.

The above conclusion does not extend to serotype A or C viruses. There is only a very slight reduction of infectivity and immunogenicity of type C-strain 997 viruses treated with trypsin (Rowlands, Sangar and Brown, 1971). Trypsinized type A_{12}-strain 119 virus is just as immunogenic as the native virus although infectivity is reduced 100–1000-fold (Bachrach *et al.*, 1975; Cavanagh *et al.*, 1977; Moore and Cowan, 1978). Recent experiments showed that absorption of A_{12}119 virus to cell membrane is abolished by trypsin (Baxt and Bachrach, 1982); hence 99·9% of the infectivity is lost (Bachrach, 1977).

The antibodies elicited by trypsinized virus, both serotypes C and O, differ from those elicited by the native virion (Rowlands, Sangar and Brown, 1971). When trypsin-treated virus was used to absorb serum induced by native virion, the neutralizing activity of the serum was only slightly diminished. In the same series of experiments, trypsinized virus induced only a low level of neutralizing antibodies, all of which could be absorbed by the trypsin-treated virus. These data suggest that the complete virion induces at least two classes of neutralizing antibodies: the major class neutralizes the intact virion and the minor type neutralizes only the trypsinized virus.

VP1—THE PROTEASE-SENSITIVE POLYPEPTIDE

From the trypsin-sensitive properties of O_1K viruses, where the infectivity and immunogenicity decrease dramatically, it was reasoned that the cell attachment and immunogenic sites are present on the surface of the virion. Electron-microscopy studies showed that neutralizing IgM antibodies bind specifically to the vertices of native virions, but not to trypsin-treated virus (Brown and Smale, 1970). Although there is no gross observable alteration (e.g. size, morphology, sedimentation constant and density in caesium chloride) in the

virus treated with trypsin or chymotrypsin, the treated viruses do not react with neutralizing antibodies in immunodiffusion experiments, and migrate considerably faster in electrophoretic analysis (Wild, Burroughs and Brown, 1969; Brown and Smale, 1970; Meloen, 1976). When intact virions were iodinated to identify surface proteins (Laporte and Lenoir, 1973; Talbot *et al.*, 1973), more than 90% of the label was associated with VP1, even though in strain O_1K, VP2 and VP3 have twice as many tyrosines as VP1 (Bachrach, Swaney and Vande Woude, 1973). When the proteins of trypsin-treated (Wild and Brown, 1967; Talbot *et al.*, 1973) and chymotrypsin-treated (Cavanagh *et al.*, 1977) O_1K viruses were analysed on PAGE, VP1 was found to be cleaved into two lower molecular weight peptides, whereas VP2, VP3 and VP4 remained unaltered. Trypsinized $A_{12}119$ viruses also gave a similar PAGE pattern with VP1 being cleaved into two 16 kd polypeptides (VP1a and VP1b). It appears that trypsin cleaves VP1 only once in both cases and the effect of proteolytic enzymes on the infectivity and immunogenicity is strain specific.

VP1—THE IMMUNIZING POLYPEPTIDE

It was first reported by Laporte *et al.* (1973) that VP1 carries the major antigenic determinants of FMDV. Of the four purified viral capsid proteins, only VP1 was able to induce neutralizing antibody in guinea pigs.

This observation was confirmed by Bachrach *et al.* (1975) using the $A_{12}119$ virus. When guinea pigs were vaccinated twice with 100 micrograms (μg) of VP1, VP2 or VP3 or with 5 μg of AEI-inactivated serotype $A_{12}119$ virus, protective antibodies were present only in animals that received VP1 or AEI-inactivated virus. Protective antibodies were not observed in animals immunized with VP2 or VP3. The anti-VP1 sera were type specific since they did not precipitate type O1 or C3 viruses. The titres of the anti-VP1 sera were lower than antiviral sera (0·5–2·8 vs. 2·6–3·4) even though a greater amount of VP1 polypeptide was used. Qualitatively, the anti-VP1 sera recognized fewer antigenic determinants than did the antiviral sera in immunodiffusion precipitation analyses. No precipitin line was evident between anti-VP1 serum and the 12S particles, although a reaction between the antiviral serum and the 12S particles was seen. Apparently, the major antigenic determinants on VP1 are different whether they are present in the viral particle, 12S particle or the isolated protein.

Experiments in which swine were immunized either with VP1 or with VP1, VP2 and VP3 each at a separate site of the same animal, gave the same titre of neutralizing antibody and the same degree of protection after three inoculations (Bachrach *et al.*, 1977). When a mixture of VP1, VP2 and VP3 was used for vaccination, a lesser degree of protection was observed. The authors' explanation is that, upon mixing the three structural polypeptides, intermolecular association occurs and the antigenic determinants on VP1 are perturbed. Experiments with larger groups of swine (Bachrach *et al.*, 1977) showed that one vaccination with 100 μg VP1 is not sufficient to impart adequate protection to the animals (one out of 10), whereas two vaccinations significantly increased the degree of protection (eight out of 10). Although the AEI-treated virus is a more potent immunogen (five out of five swine were fully protected when

vaccinated once with 5 μg of inactivated virus in the same experiment), it is possible to protect swine against FMDV with VP1 after multiple injections.

IMMUNIZING ACTIVITY OF VP1 FRAGMENTS

Kaaden, Adam and Strohmaier (1977) showed that cleavage fragments of VP1 are capable of inducing neutralizing antibodies which can protect animals against FMDV. Cyanogen bromide cleaves O_1K VP1 into five fragments, each of molecular weight less than 14 kd. After four injections of approximately 100 μg of either intact or cyanogen-bromide-cleaved VP1 of O_1K, guinea pigs showed similar levels of neutralizing antibody titre with both antigens; however, the level is much lower than that elicited by native virions. Upon challenge with virulent virus, only a fraction of the animals vaccinated with either the intact VP1 or the cyanogen-bromide-cleaved polypeptide proved to be resistant to the disease.

Similar experiments using the $A_{12}119$ virus revealed that the 13 kd peptide generated by cyanogen bromide cleavage of VP1 is the major peptide responsible for eliciting neutralizing antibodies in guinea pigs (Bachrach, Morgan and Moore, 1979). These studies demonstrated that the complete VP1 polypeptide is not necessary for the induction of neutralizing antibodies against FMDV. It is possible to use VP1 fragments for vaccination as long as they present the immunogenic determinants in a form recognized by the immune system.

In summary, all the experiments described above show that VP1 is the only polypeptide of FMDV capable of inducing neutralizing antibodies. This protein seems to carry all the antigenic determinants necessary to protect animals against infection, and therefore makes a good candidate for the application of recombinant DNA technology in vaccine production.

Application of biotechnology

CLONING OF VP1-SPECIFIC SEQUENCES IN *ESCHERICHIA COLI*

The introduction of genetic materials of exogenous origin into a prokaryotic host by recombinant DNA techniques has become part of the routine repertoire of a molecular biology laboratory. A specific gene can be isolated, and then propagated and studied in a 'controlled' environment.

With the realization that isolated VP1 could protect animals against FMD (LaPorte *et al.*, 1973; Bachrach *et al.*, 1975; Meloen, Rowlands and Brown, 1979), the advantage of a subunit vaccine over conventional vaccine became self-evident. A subunit vaccine produced by recombinant DNA is absolutely safe because neither the viral particle nor the viral RNA is involved in any step of vaccine production. Furthermore, in comparison with conventional methods, larger quantities of antigens can be manufactured more quickly and economically.

By 1981, three groups had succeeded in cloning and determining the nucleotide sequences of DNA copies of the genomes of three different FMDV strains; subsequently, two groups were able to express the VP1 coding sequences in *E. coli*. The same standard complementary DNA (cDNA) cloning procedures

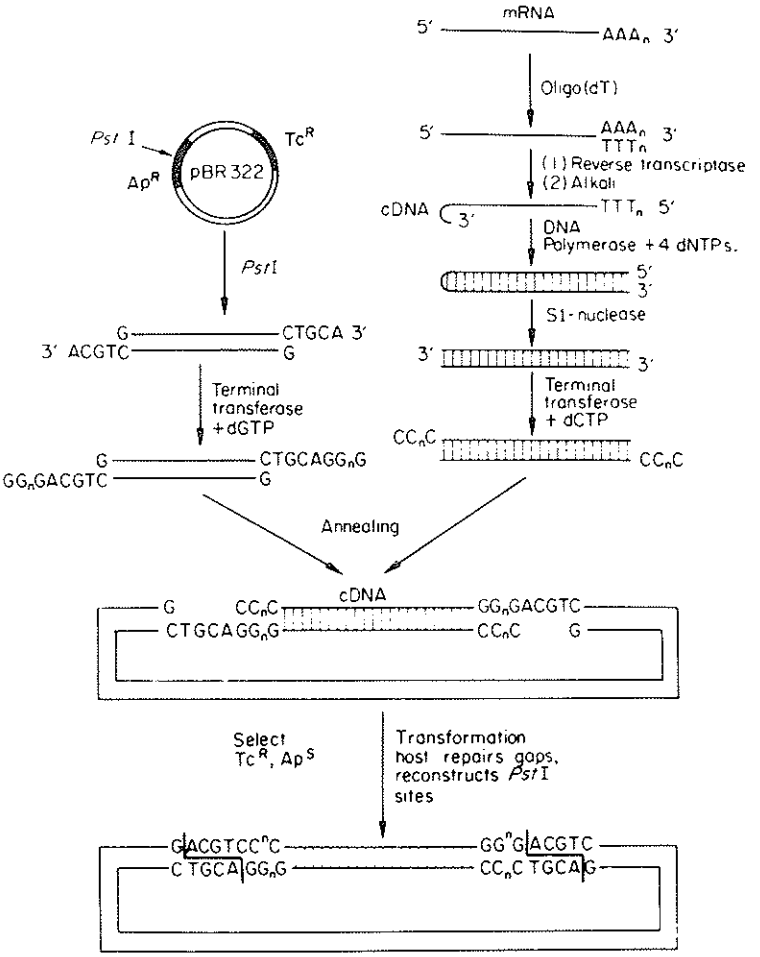


Figure 2. Schematic diagram of cDNA cloning. *See text* for details.

were used by all the investigators to produce bacteria that contain FMDV sequences. The general method for introducing foreign DNA sequences into a bacterial cell is outlined in *Figure 2*. Küpper *et al.* (1981) were the first to report the cloning and expression of FMDV VP1 cDNA sequences of O_1K. Their procedures and results are used as a prototype in the present review. The vector used was plasmid pBR322 which carries resistance markers for ampicillin and tetracycline. The *Pst*I endonuclease restriction site located within the β-lactamase gene was selected for the introduction of double-stranded (ds) cDNA sequences by the G:C tailing method (Villa-Komaroff *et al.*, 1978).

Since the genetic material of FMDV is a single-stranded RNA, a ds-cDNA copy has to be produced in order to introduce FMDV specific sequence into *E. coli*. The usual process is to prime the first strand cDNA with oligo(dT), which is complementary to the poly (A) tail at the 3′ end of the genomic RNA. DNA synthesis is carried out by reverse transcriptase. After removal of the viral RNA, the cDNA folds back on itself and thus primes synthesis of the second cDNA

strand by DNA polymerase I. The hair-pin structure at the 5′ end of the ds-cDNA is cleaved with S1 nuclease and oligo(dC) tails are then added to both ends of the ds-cDNA molecule. These molecules are annealed to *Pst*I cut plasmid pBR322 DNA which had had oligo(dG) tails added to its ends. After annealing, the mixture is used to transform competent *E. coli* host cells. Because insertion of DNA at the *Pst*I site inactivates the β-lactamase gene responsible for ampicillin resistance, tetracycline-resistant and ampicillin-sensitive bacterial colonies were selected for further analysis. Thus a FMDV strain O_1K-specific cDNA library was established.

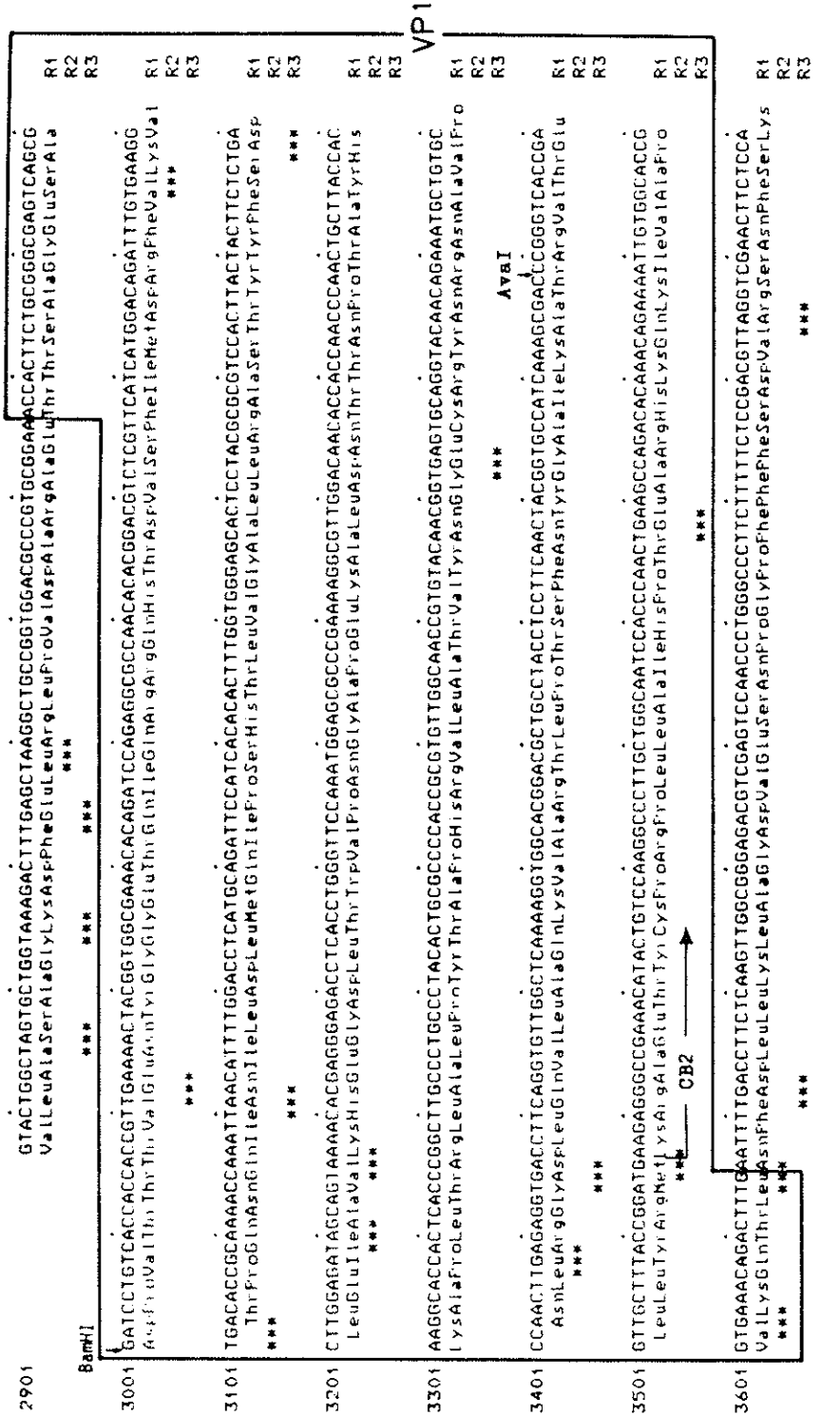
So far, none of the investigators have reported the cloning of the entire FMDV genome as a contiguous cDNA sequence in *E. coli*. Rather, they all obtained inserted fragments of variable length (up to 4000 base pairs) covering various parts of the viral genome (*Figure 1b*). In particular, the poly (C) track or sequences 5′ to the poly (C) track were not observed in any of the clones. It appears that the long homopolymer region is unclonable by the conventional methods described above. Consequently, the chance of generating infectious RNA or DNA molecules by introducing the entire FMDV genome into a bacterium appears highly unlikely.

IDENTIFICATION OF THE VP1-SPECIFIC SEQUENCE

Initially, Küpper *et al.* (1981) constructed a physical map (*Figure 1b*) of the FMDV genome based on the size and intensity of the DNA fragments generated by digestion of ^{32}P-labelled ds-cDNA with various restriction enzymes. Assuming that the cDNA sequences corresponding to the 3′ end of the viral RNA will occur more frequently because of the oligo(dT) priming method, the restriction fragments were ordered. This physical map from restriction enzyme analyses was aligned with the known biological map constructed from the order of translation and the molecular weights of the viral polypeptides (Doel *et al.*, 1978). By this comparison, the VP1 coding sequence was predicted to be present in a 850 base pair *Bam*H1/*Hin*dIII fragment which corresponds to a region of the viral RNA located 5000 nucleotides from the poly (A) tail. DNA fragments covering this area of O_1K (FMDV −115, −144 and −1034) were first analysed for their nucleotide sequence by the method of Maxam and Gilbert (1977).

By that time, the first 40 *N*-terminal amino acid residues of O_1K VP1 polypeptide had been elucidated (Adam and Strohmaier, 1974; Strohmaier, Wittmann-Liebold and Geissler, 1978). Computer analysis was then conducted to locate these 40 residues within the deduced amino acid sequences from the cDNA fragments; thus the coding sequence of capsid protein VP1 was located (Küpper *et al.*, 1981). Consequently, the complete VP1 coding sequence was established.

As the primary translation product from the viral RNA is a polyprotein from which the viral capsid proteins are derived, the correct reading frame should allow complete read-through of the nucleotide sequence. The reading frame was unequivocally established since only one frame allows translation without interruption, whereas the other two frames contain multiple termination codons (Kurz *et al.*, 1981; *Figure 3*).


VP1
BamHI
AvaI
CB2
R1
R2
R3

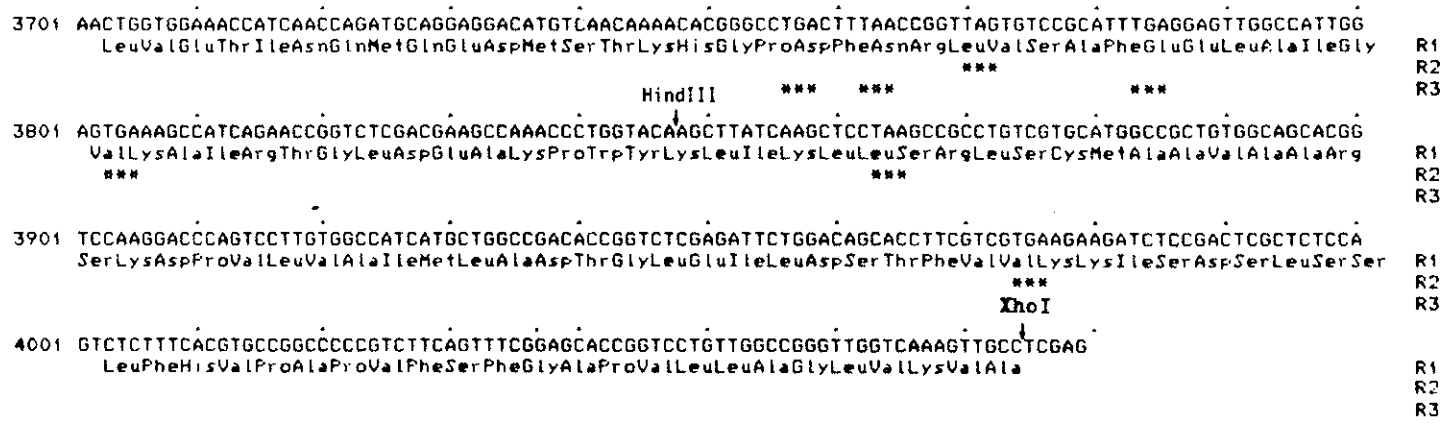


Figure 3. Nucleotide sequence of cloned FMDV–O_1K cDNA. The deduced amino acid sequence of the translation product is shown for the first phase, potential stop codons (***) in the other two phases. The sequence that codes for VP1 is boxed. (Courtesy of Kurz *et al.*, 1981).

With the current amount of FMDV sequence data available, the identification of VP1-specific sequences of any desired strain is relatively easy. Comparison of data from several FMDV strains showed that the 3′ flanking sequences, i.e. those coding for the P52 protein, are highly conserved. Cheung *et al.* (1983) have successfully used sequences from this region as probes to identify VP1-containing clones of other FMDV strains.

DETERMINATION OF THE GENE BOUNDARIES OF VP1

The amino acid sequence derived from the DNA sequence was compared with the partial amino acid sequence of VP1 obtained by protein sequencing (Strohmaier, Wittmann-Liebold and Geissler, 1978). Except for discrepancies at positions 7 and 33, the derived protein sequence is identical to the peptide-sequencing results. Thus the *N*-terminal boundary of VP1 could be assigned to the appropriate nucleotide sequence. The carboxyl-terminal boundary was determined by digestion both of the purified VP1 and of the *C*-terminal fragment of the cyanogen-bromide-cleaved VP1 with carboxypeptidase Y (EC 3.4.16.1). Leucine, threonine and glutamine were preferentially released in that order. In conjunction with the estimated molecular weight of VP1, the carboxyl end, Glu-Thr-Leu, was assigned to the corresponding codons (Kurz *et al.*, 1981; Strohmaier, Franz and Adam, 1982). Thus the VP1 polypeptide of O_1K contains 213 amino acid residues.

The VP1 gene boundaries for $A_{12}119$ were determined in an analogous way. The *N*-terminal residues for $A_{12}119$ were reported to be Thr-Thr-Ala-Thr (Matheka and Bachrach, 1975). Subsequent experiments extended this sequence to the first 26 amino acid residues (Bachrach, Morgan and Moore, 1979). Although there is a discrepancy between the deduced and the directly obtained amino acid sequences, there is enough agreement to assign the first codon of the $A_{12}119$ VP1 polypeptide. Since the $A_{12}119$ *C*-terminal residues, Gln-Thr-Leu, obtained by direct amino acid determination were available (Bachrach, Swaney and Vande Woude, 1973), the *C*-terminal codon could be designated. Thus $A_{12}119$ VP1 polypeptide contains 212 amino acid residues.

Both the *N*- and *C*-terminal gene boundaries for VP1 of $A_{10}61$ were first estimated based on the partial amino acid sequences published for other FMDV strains as described above. Subsequently, the *N*-terminal amino acid residues were confirmed by direct sequencing (Boothroyd *et al.*, 1981, 1982). The VP1 of $A_{10}61$ contains 212 amino acid residues.

Today, due to the availability of enough sequence information, the newly derived VP1 sequences of additional FMDV strains can be directly aligned with the already established boundaries by analogy.

EXPRESSION OF THE VP1 SEQUENCE IN *E. COLI*

In order to produce large quantities of a given protein, its synthesis in a fast-growing bacterium such as *E. coli* is desirable. The regulation of gene expression in this organism is well understood. Once the structural gene of the protein is isolated, it can be placed under the control of prokaryotic sequences required for efficient transcription and translation.

In general, it is desirable to regulate the expression of the inserted coding sequence because the expressed protein might be toxic to the cell and, therefore, interfere with cell growth even when the gene product is present at low intracellular concentration. Of the many regulated promoters known, two have been applied successfully for larger-scale fermentation of genetically engineered bacteria containing VP1 of FMDV: (1) the pL promoter of bacteriophage lambda which is inducible by high temperature if the cell contains a temperature-sensitive mutant repressor protein; (2) the bacterial *trp* promoter which can be induced by tryptophan starvation. Both of these are strong promoters and can be easily regulated. If the pL promoter is used, the corresponding repressor protein has to be present in the host cell. The gene for the repressor protein can be present either on the chromosome or on a separate plasmid.

No matter how strong the promoter, the final production of the proteins is further dependent on efficient translation of the mRNA and the stability of the product. In a number of cases, it appears that proteins which are naturally secreted are unstable inside the cell. One possible explanation for this is that the reducing conditions inside inhibit the proper folding of the protein which normally occurs in the relatively more oxidizing conditions outside the cell; the protein thus becomes an efficient substrate for the host's degradation mechanisms. Sometimes the problem of stability can be overcome by fusing the protein to part of a bacterial protein. This method of stabilization has been successfully applied to somatostatin (Itakura *et al.*, 1977) by fusing the gene for the small hormone to that of β-galactosidase; the relatively large fusion protein which resulted was resistant to degradation by bacterial proteases.

To express the VP1 gene of FMDV strain O_1K, Küpper *et al.* (1981) chose an expression vector which had already been used to express the human fibroblast interferon gene (Derynck *et al.*, 1980). This plasmid (pPL c24, *Figure 4*) carries the strong pL promoter together with the DNA sequence coding for the first 99 amino acids of bacteriophage MS2 replicase, followed by unique sites for the restriction endonucleases *Bam*H1 and *Hin*dIII. Using these two sites, the 847 bp *Bam*H1/*Hin*dIII fragment of clone pFMDV-1034 (*Figure 1b*) was inserted into the vector where only one orientation is possible. The insertion places the coding sequence of FMDV, starting with amino acid 9 of capsid protein VP1, in phase with the MS2 replicase; thus the ribosome binding site and the beginning of the replicase gene are used to initiate translation of the VP1 gene, which as a processing product does not have its own initiation signals. Translation should produce a fusion protein of 396 amino acids beginning with the 99 *N*-terminal amino acids of the MS2 replicase, followed by 284 residues specific for FMDV and then 13 residues encoded by the vector. The inserted FMDV fragment codes for 205 amino acids of VP1 plus the first 79 amino acids of protein P52, the gene for which is adjacent to the gene for VP1 in the FMDV genome (*Figure 1a*). This plasmid (pPL-VP1, *Figure 4*) was transformed into an *E. coli* C600 strain which already carried a gene specifying the temperature-sensitive lambda repressor on a second plasmid, pcI857, carrying the kanamycin resistance gene (Remaut, Stanssens and Fiers, 1981). This allowed the expression of the pL promoter to be controlled by temperature. Cultures grown at 28°C do not make the fusion protein. When the temperature is shifted to 42°C the

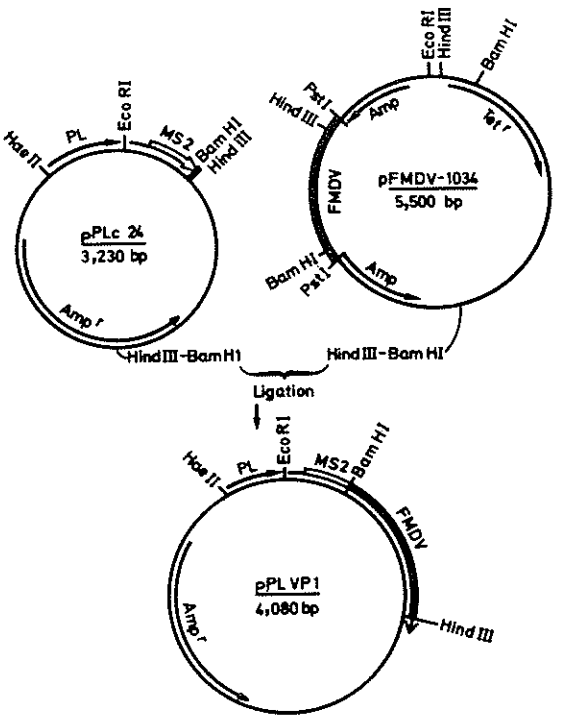


Figure 4. Construction of plasmid pPL VP1. The schematic outline shows the vector plasmid pPL c24 and the donor plasmid pFMDV-1034. Amp: β-lactamase region coding for resistance to ampicillin; Tet: coding region for resistance to tetracycline; PL: λ P_L control element; MS2: DNA fragment derived from bacteriophage MS2 where the boxed part indicates the coding sequence for the first 99 amino acids of the MS2 polymerase; FMDV: insert derived from FMDV cDNA, the shadowed area shows the region coding for VP1. The predicted fusion polypeptide from the constructed plasmid pPL VP1 is indicated. Only restriction sites relevant for this construction are shown. (Courtesy of Küpper *et al.*, 1981).

repressor is inactivated and expression of a 43 kd protein can clearly be demonstrated by SDS-PAGE (*Figure 5*). This protein accounts for up to 20% of the total cellular protein, which is equivalent to about 1×10^6 molecules per bacterial cell (Küpper *et al.*, 1982). The newly synthesized protein was identified as the MS2-VP1 fusion protein by its reaction with murine antibodies against capsid protein VP1. This test shows that, indeed, the correct protein is synthesized in larger amounts and that the protein is not degraded inside the bacterial cell. When smaller portions of the MS2 replicase (e.g. only the first 15 amino acids) were used, the amount of protein found in the cell was reduced 20-fold (Küpper, unpublished observation). It is possible that the hydrophobic part of the MS2 replicase stabilizes the fusion protein by forming a precipitate within the cell and thus making it insoluble and inaccessible to cellular proteases.

Phase contrast microscopy and thin-section electron microscopy revealed that intracellular inclusion bodies are present in bacteria (C600 (pcI857)/pPL-VP1)

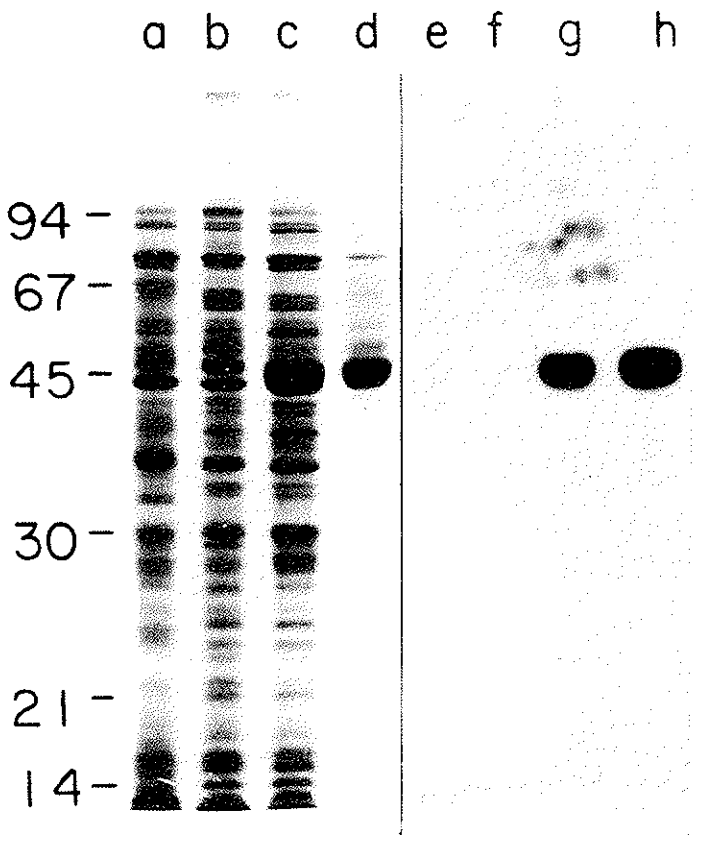


Figure 5. Expression of cloned FMDV–O_1K VP1 gene. SDS–PAGE of total cellular proteins. (a) C 600 (pcI 857) induced for 3 hours at 42°C; (b) C 600 (pcI 857)/pPL VP1 not induced, and (c) induced for 3 hours at 42°C. (d) Proteins extracted from the membrane pellet by 6 M urea. Lanes (e–h) are autoradiograms of same proteins as lane (a–d) but transferred to nitrocellulose and reacted with ^{125}I-labelled VP1-specific antibodies. (Courtesy of Küpper *et al.*, 1982).

that produce large quantities of the fusion protein but absent from cells not carrying the expression plasmid (*Figure 6*).

Such electron-dense inclusion bodies have been observed before in genetically manipulated bacterial cells producing a fusion protein consisting of β-galactosidase and human insulin (Williams *et al.*, 1982).

Hardy, Stahl and Küpper (1981) transferred the O_1K VP1 gene from the pPL-VP1 expression plasmid into the *Bacillus subtilis* pBD9 plasmid. In this way, VP1 is expressed in *Bacillus subtilis* as a chimeric protein containing 73 amino acid residues encoded by the erythromycin resistance gene, 205 residues by FMDV VP1, 79 residues by P52 and 13 residues by the bacterial vector. The fusion protein represented about 2% of total cell protein and was stable in the *Bacillus* host.

Kleid *et al.* (1981) inserted a ds-cDNA copy of the subtype $A_{12}119$ genome into the *Pst*I site of pBR322 and then transferred the VP1 cDNA sequences to a site in an expression vector downstream of a region containing the trypto-

Figure 6. Electron micrograph of *E. coli* carrying pPL-VP1 that expressed VP1 fusion protein at high levels. 537 is the parent bacterium C600(pCI857) and 537/pPL-VP1 carries the expression plasmid for FMDV VP1. Both preparations were induced at 42°C.

phan promoter, the *trp* leader (L) gene and part of the *trp* E protein gene. This plasmid was transformed into *E. coli* and propagated in low concentration of tryptophan. When tryptophan in the medium is depleted, the tryptophan promoter is de-repressed and allows expression of a 394-residue VP1 fusion protein. This protein contains 190 amino acid residues from the (L + E) sequences and 204 amino acids of VP1 (the 6 *N*-terminal and the last *C*-terminal amino acids are missing). This chimeric protein is insoluble inside the host cell and is found in amounts corresponding to approximately 17% of total *E. coli* protein by SDS-PAGE. The protein reacts with anti-VP1 ($A_{12}119$) antibody.

PURIFICATION OF THE BACTERIAL VP1 FUSION PROTEIN

To prepare VP1 fusion protein for vaccination, it is necessary to extract the antigen from the bacterial host.

The bacterial strain C600 (pcI857)/pPL-VP1 is grown in L-broth containing ampicillin and kanamycin at 28°C overnight (approximately $A_{550\,nm} = 3$). Next day, the culture is diluted fourfold into a pre-warmed medium (42°C) and further incubated for 3 hours. During this time the cells divide once and accumulate approximately 20% of total cellular proteins as the VP1 fusion protein. The cells are collected by centrifugation, resuspended in phosphate-buffered saline (PBS) and broken by sonication. Because of the insoluble nature of the fusion protein, all soluble proteins can be washed away by simple washing procedures, leaving the desired protein and membrane particles in the precipitate. The VP1 protein is extracted from this pellet with high concentrations of urea to give a product of more than 50% purity (*Figure 5, lane d*). The protein is further purified by column chromatography, but must be kept in urea buffer to prevent aggregation and precipitation. These steps involved in the production of the VP1-fusion protein can be carried out on large volumes of cells. In fact, production in fermenters often gives better results than shake flask experiments because bacterial growth can be optimized by monitoring the pH and oxygen concentration of the culture.

EFFICACY OF BACTERIAL-PRODUCED VP1 IN ANIMALS

Although several laboratories are actively engaged in research in this field, relatively few animal experiments of the genetically engineered vaccine have been reported. This is because the vaccine has become available only recently and the animal experiments are time-consuming.

Kleid *et al.* (1981) injected LE-VP1 ($A_{12}119$), purified by SDS-PAGE, into cattle and swine using an oil adjuvant. Neutralizing antibodies were detected after two injections (250 μg each). This immunization procedure protected both cattle (five out of six fully protected, one animal with minor foot lesions) and swine (two out of two); non-vaccinated control animals (two cattle and two swine) came down with severe foot and mouth lesions (*Table 1*). Hofschneider *et al.* (1981) immunized goats with crude bacterial extracts of MS2-VP1 fusion protein (O_1K) and found that two out of three goats had anti-VP1 antibodies when tested by enzyme-linked immunosorbent assay (ELISA). When a purer preparation of MS2-VP1 was used for immunization, two out of three goats produced neutralizing antibodies. Upon challenge both goats had less fever and lower viraemia than non-vaccinated control animals. The most successful fusion-protein vaccine experiment until now has been the immunization of cattle using the LE-VP1 fusion protein of $A_{12}119$ by J. J. Callis *et al.* (personal communication). After two inoculations of 10, 50, 250 or 1250 μg each of fusion protein, five, seven, eight or nine out of nine cattle could be protected, respectively (*Table 2*).

It was also reported that high neutralizing antibody titres could be elicited in guinea pigs by a single injection of 100 μg of the LE-VP1 fusion protein

Table 1. Neutralizing antibody and immune responses of cattle and swine vaccinated with LE′–VP3 fusion protein or VP3 isolated from virus. VP3 of this table is identical to VP1 of this review. (Courtesy of Kleid *et al.*, 1981).

		Neutralizing antibody day after vaccination*								Challenge of immunity†		
Subject	Antigen	7	14	21	28	35	42	46	56	Mouth lesions	Foot lesions	VIA antibody‡
Cattle												
1	LE′–VP_3	<0·3	0·5	0·3	1·2	2·3	2·7		2·9	0	0	–
2	LE′–VP_3	0·2	0·9	1·3	2·0	2·4	2·7		2·9	0	0	–
3	LE′–VP_3	0·2	0·4	1·1	1·7	1·9	2·1		2·9	0	0	–
4	LE′–VP_3	0·2	0·7	0·7	1·1	2·2	2·6		2·9	0	0	–
5	LE′–VP_3	<0·3	0·9	1·0	1·1	2·6	2·5		2·6	0	+	–
6	LE′–VP_3	<0·3	1·0	1·3	1·3	2·1	2·1		2·3	0	0	–
7	VP_3	0·1	1·0	2·1	1·9	2·2	2·6		2·1	0	0	–
8	VP_3	<0·3	0·4	0·4	0·7	1·7	1·6		4·1	+ + +	+ + +	+
9	None†								3·4	+ + +	+ + +	+
10	None								3·5	+ + +	+ + +	+
11	None								3·3	+ + +	+ + +	+
12	None								3·3	+ + +	+ + +	+
Swine												
13	LE′–VP_3	<0·3	<0·3	0·5	1·5			3·0		0	0	–
14	LE′–VP_3	<0·3	0·3	0·6	1·4			3·0		0	0	–
15	None†							3·5		+ + +	+ + +	+
16	None							3·4		+ + +	+ + +	+

* Revaccinations on day 24 for swine and day 28 for cattle; titre is $-\log_{10}$ of serum dilution that protects 50% of suckling mice. The 46- and 56-day sera were collected 14 days after challenge.
† Challenged on day 32 for swine and day 42 for cattle. Non-vaccinated animals 9 to 12 and 15 and 16 constituted the challenge groups; half were inoculated with virulent type A_{12}119ab virus and half were contact transmission controls; +, single small lesion, not generalized disease; + + +, numerous lesions and generalized infection.
‡ Presence, +, of virus-infection-associated antigen (VIA) antibody in sera collected 14 days after challenge indicated animals experienced FMD; –, VIA antibody absent.

Table 2. Neutralizing antibody and immunity in cattle vaccinated with biosynthetic A12 VP1 vaccine. (Courtesy of J. J. Callis *et al.*, personal communication).

	Weeks after vaccination											
Antigen (μg)	2	8	12	15	17	21	30	32	34	38	42	45
10	0·9	0·9	0·9	1·7†	2·0*	1·7	1·7‡	—	—	—	—	—
50	1·0	1·2	1·0	1·8†	2·1*	1·9	2·0‡	—	—	—	—	—
250	1·1	1·1	1·0	2·0	2·0	1·8	1·8	1·9†	2·3*	2·6	2·5	2·4‡
1250	1·2	1·3	1·3	2·0	2·6	2·0	1·9	2·3	1·9*	2·9	2·4	2·7

* Titre 2 weeks after revaccination.
† Revaccination with 10, 50, 250, or 1250 μg dose respectively.
‡ Challenge of immunity: 10 μg, 30 weeks, 5/9 immune; 50 μg, 30 weeks, 7/9 immune; 250 μg, 45 weeks, 8/9 immune; 1250 μg, 45 weeks, 9/9 immune.

(Moore, 1983; *Table 3*). This result is very important, because the efficacy of FMDV vaccines is currently judged by their ability to elicit a high titre of neutralizing antibody after a single injection. However, in a parallel experiment with the O_1Campos strain, injection of 500 μg of a LE-VP1 fusion protein did not give as high a neutralizing antibody titre as the fusion protein from A_{12}119. This is another indication that serotype O VP1 polypeptide is intrinsically less immunogenic (as described above), whether it is present as a fusion protein or in the virion when compared with serotype A. In addition, the same series of

Table 3. Neutralizing antibody responses of guinea pigs LE'–VP3 fusion proteins or FMDV VP3. VP3 of this table is identical to VP1 of this review (Courtesy of Moore, 1983).

Group	Antigen	Buffer†	Neutralizing antibody response at given dose (μg)			
			4·0	20	100	500
1	FMDV $A_{12}VP_3$	Urea–Tris	3·2	3·9	4·8	—
2	Long leader $A_{12}LE'–VP_3$	Urea–Tris	3·5	3·9	3·6	—
3	Short leader $A_{12}LE'–VP_3$	Urea–Tris	2·3	3·6	3·6	—
4	Short leader $A_{12}LE'–VP_3$	Gel slurry SDS–Tris	<0·3	1·7	4·2	—
5	Short leader $A_{12}LE'–VP_3$	Urea–Tris	—	—	3·2	3·6
6	Short leader $O_1LE'–VP_3$	Urea–Tris	—	—	1·5	2·2
7	Short leader $O_1LE'–VP_3$	Urea–Tris	—	—	1·8‡	3·2‡
8	Long leader $A_{12}LE'–VP_3$ tandem peptide Amino Acids #137–167	Urea–Tris	—	—	4·6§	—

* Neutralizing antibody titres determined by the suckling mouse serum neutralization test (Skinner, 1952) of guinea pig sera, taken 28–35 days after vaccination. Values are the $-\log_{10}$ serum dilution 50% end-point of a pool of 5 sera protecting mice from 100 LD_{50} doses of the homologous virus. For vaccination appropriate doses in a 2 ml aqueous-Freund incomplete oil adjuvant emulsion were given subcutaneously in the skin of the neck. — indicates not tested.

† Indicates the aqueous phase of the vaccines: Urea–Tris = 6 M urea, 0·05 M mercaptoethanol 0·014 M Tris–HCl, pH 8·6; gel slurry SDS–Tris = pulverized polyacrylamide preparative gel slice diluted in 0·1% SDS, 0·05 M Tris–glycine, pH 8·1.

‡ Neutralizing antibody titre 21 days after revaccination at 35 days with the same vaccine preparation.

§ Neutralizing antibody titre on day 28, revaccinated on day 14 with same preparation.

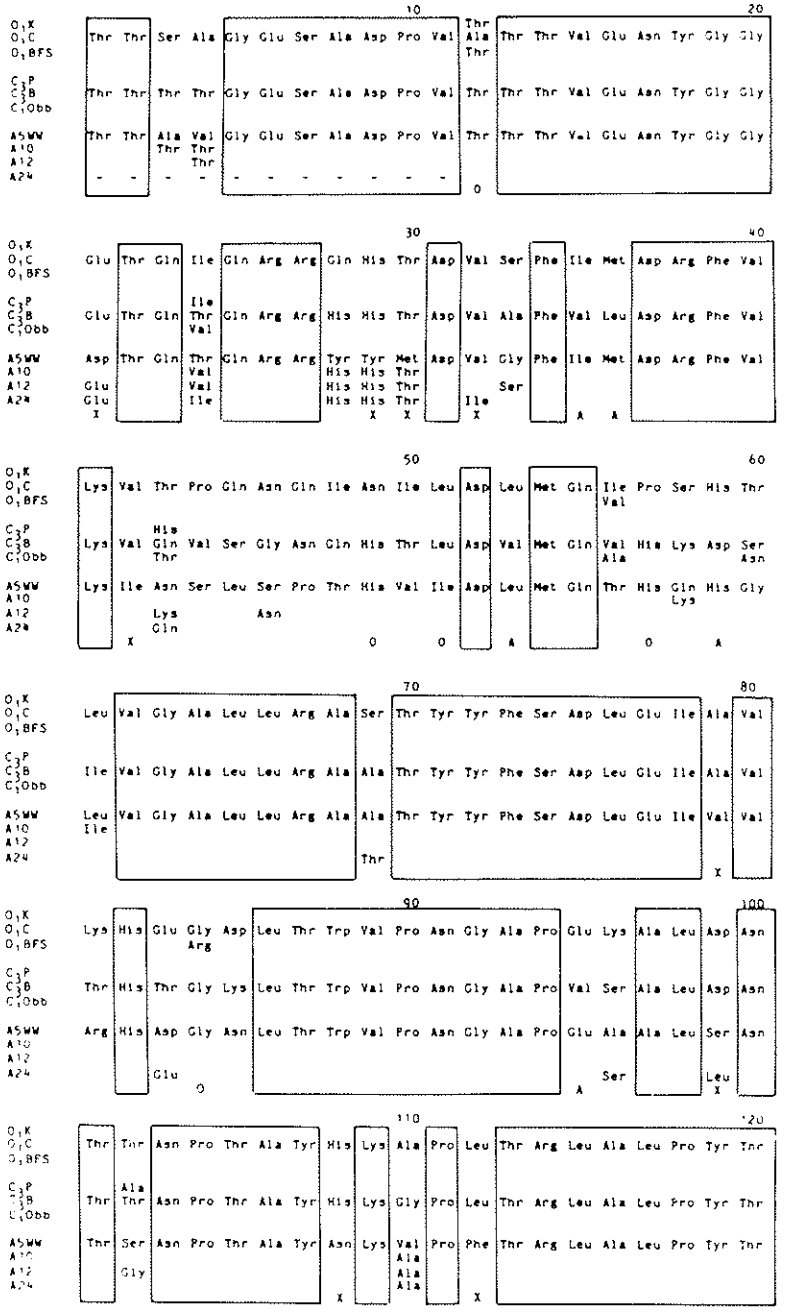
experiments showed that the length of the leader amino acid sequence does not affect the immunogenicity of the fusion protein.

In all the experiments reported, the 'vaccine' was freshly prepared in urea–tris buffer or with acrylamide particles present. However, for a commercial vaccine, it is necessary to formulate the vaccine with the appropriate adjuvants, e.g. oil, aluminum hydroxide or others, acceptable to the veterinarians in the field. Further experimentation is required to assess the duration of protection imparted to the animals, the shelf-life under various climatic conditions and the side-effects, if any. In addition, it should be stressed that all fusion proteins tested to date have employed protein of only one serotype at a time, whereas all commercial vaccines now in use are multivalent.

Large field trials have not yet been reported for the biochemically engineered vaccine. However, if extensive research, especially in the field of vaccine formulation, shows encouraging results the fusion protein will be tested in the field in the near future.

The above summary has shown that large quantities of VP1 fusion proteins can be produced in bacteria. The chimeric polypeptides are stable, with no detectable changes in molecular weight or antigenicity when treated for 10 minutes at 100°C in the presence of denaturing agents, e.g. β-mercaptoethanol and SDS (Kleid *et al.*, 1981; Küpper *et al.*, 1982). This genetically engineered protein is capable of protecting guinea pigs, swine, and cattle against FMD.

However, there are problems which remain unsolved. Protective antibodies elicited by viral or genetically engineered VP1 differ quantitatively and qualitatively from those elicited by the native virion (Cartwright, Chapman and Brown, 1980). On an equimolar basis, several orders of magnitude more VP1 protein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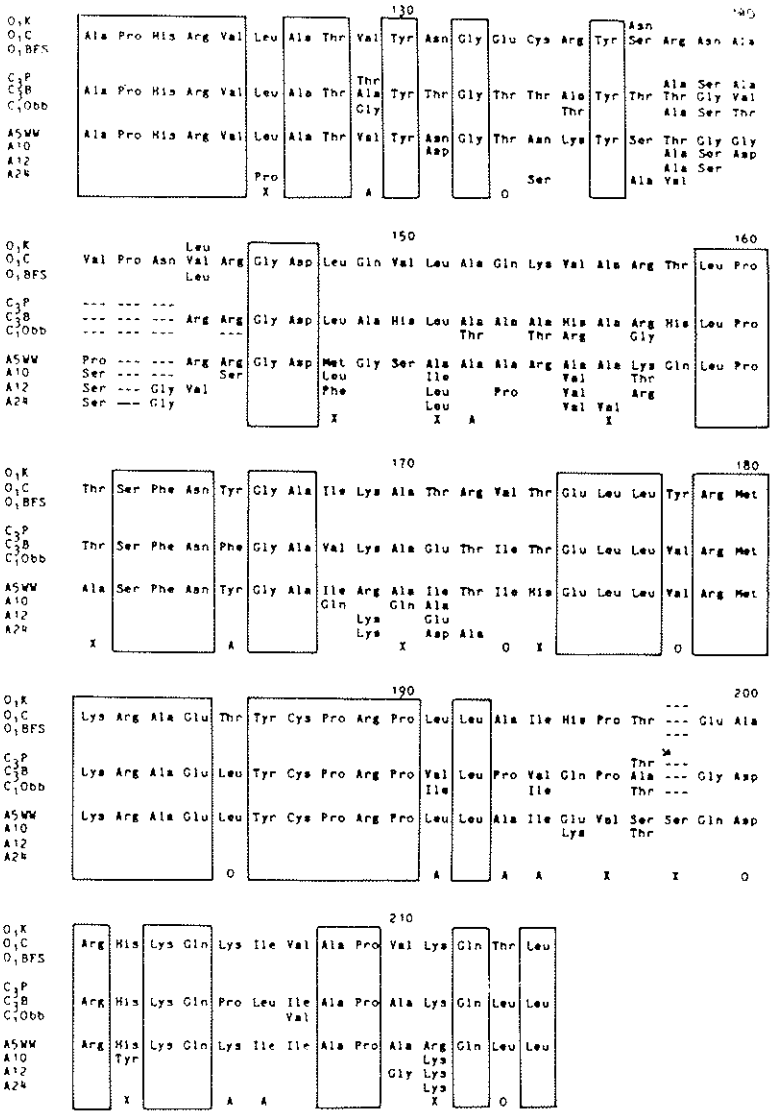

Figure 7. (Left and above). Comparison of the VP1 amino acid sequence of ten FMDV isolates. O_1Campos, C_3Indaial-B and A_5Westerwald sequences are used as prototype for comparison. Amino acid residues present in other isolates that differ from the prototype residues are replaced with the appropriate residues accordingly. Amino acids residues shared by all 10 isolates are enclosed in boxes. Additional residues common to serotypes (O and C), (C and A) and (O and A) are indicated by X, O and A respectively at the bottom of the appropriate positions, and residue deletion is indicated by (---). (Based on data of Boothroyd *et al.*, 1981; Kleid *et al.*, 1981; Kurz *et al.*, 1981; Makoff *et al.*, 1982; Beck, Feil and Strohmaier, 1983 and Cheung *et al.*, 1983).

than complete virus is required to elicit neutralizing antibodies in animals. In addition, Meleon *et al.* (1982) have demonstrated that neutralizing monoclonal antibodies raised against the native virus do not recognize the isolated VP1 polypeptide.

Oligopeptide vaccines

The ready availability of protein sequences through DNA sequencing and the progress in oligopeptide synthesis has opened a totally new approach to making vaccines chemically. For efficient application of this approach, prior knowledge of those amino acid sequences corresponding to the antigenic determinants of the pathogen are useful.

LOCALIZATION OF IMMUNOGENIC EPITOPES OF VP1

Amino acid sequence approach

As discussed above, there are seven distinct serotypes and more than 65 known subtypes of FMDV based on serological assays such as complement fixation and virus neutralizing tests (Pereira, 1977). The fact that antisera raised with some subtypes do not always neutralize other subtypes of the same serotype suggests that there is considerable variation in the antigenic determinants of different strains of FMDV.

Of all the capsid proteins, only VP1 has been shown to carry the major antigenic determinant(s) responsible for inducing neutralizing antibodies in animals. By comparing the primary amino acid sequence of VP1 of various strains, it is possible to identify the regions where the amino acid sequences differ between serotypes and subtypes. At present, sequence determination of DNA is easier and faster than sequence determination of protein; very limited amino acid sequences of the VP1 polypeptide are obtained by direct residue determination. In contrast, complete VP1 nucleotide sequences of more than 10 isolates have been reported, allowing the amino acid sequences to be deduced according to the genetic code.

The currently available VP1 amino acid sequences have been aligned for maximum homology (Cheung *et al.*, 1983; *Figure 7*). The residues common to all strains are enclosed in boxes; those shared by all isolates of any two different serotypes are indicated by A, O or X at the bottom of the amino acid residues. This alignment indicates that approximately 60% of VP1 amino acid sequence is conserved when any two FMDV strains are compared. In general, three conserved regions are present: these lie between positions 1–41, 61–130 and 159 to the carboxyl terminus. Two variable regions are located within positions 42–61 and 134–158. Since the amino acid residues present at 42–61 are highly homologous among strains of the same serotype, it has been suggested that both variable regions contribute to serotype specificity while only the 134–158 variable region is likely to be associated with subtype specificity (Makoff *et al.*, 1982; Beck, Feil and Strohmaier, 1983; Cheung *et al.*, 1983).

Polypeptide fragmentation approach

The fact that the entire VP1 is not necessary for eliciting protective antibodies in animals encouraged Strohmaier, Franze and Adam (1982) to localize the major antigenic determinants along the VP1 polypeptide. O_1K virions were incubated with various proteolytic enzymes of different specificities and the virions were then re-purified. Fragments derived from VP1 were analysed on SDS–PAGE and the amino acid residues at the *N*-terminus and the *C*-terminus of each fragment were also determined. In conjunction with the amino acid sequence deduced from DNA sequence analysis, the cleavage sites and peptide fragment length can be determined unequivocally.

The results showed that trypsin (EC 3.4.21.4) cleaves O_1K at multiple sites, a finding that helps to clarify results from earlier investigations of the effect of proteases on infectivity (*above*). Although the virion remains visually intact, two small peptide regions (residue positions Asn^{139}–Lys^{154} and His^{202}–Leu^{214}) are cleaved off, and the infectivity and immunizing activity of the trypsin-treated O_1K virus is reduced by 100–1000-fold (as described above). Mouse submaxillary gland protease (MSGP; endoproteinase Arg-C) cleaves VP1 between Arg^{145} and Gly^{146} but there is no loss of any amino acid residues, and the infectivity of the virion is not affected. Initially, endoproteinase Lys-C nicks O_1K VP1 between Lys^{154}–Val^{155} and a fragment containing the Gln^{204}–Leu^{214} is cleaved from the virion. At this point, infectivity of the Lys-C treated virus is not affected. Upon further incubation, the large fragment (i.e. Thr^{1}–Lys^{154}) is shortened from 21 kd to 20 kd and the infectivity of the virion is reduced. A similar result was obtained with chymotrypsin (EC 3.4.21.1) (Cavanagh *et al.*, 1977). Here, the ability of virus to attach to host cells as well as its immunogenicity was greatly reduced after long periods of digestion. Collectively, these results showed that the loss of amino acid residues between Asn^{139} and Lys^{154} destroys the ability of the O_1K virus to attach to cells.

On the basis of the immunogenic properties of the peptide fragments generated by various procedures, Strohmaier, Franze and Adam (1982) localized two potential immunogenic sites on VP1 of O_1K. The peptide fragments Gln^{55}–Arg^{179} and Lys^{181}–Leu^{214} (generated by cyanogen bromide cleavage) and the Gly^{146}–Leu^{214} (generated by MSGP digestion) can elicit significant levels of neutralizing antibodies; a small peptide, Val^{154}–Arg^{201} (generated by trypsin digestion) does not possess any immunogenic activity. This suggests that Gly^{146}–Lys^{154} and His^{202}–Leu^{214} are the only non-overlapping amino acid sequences in VP1 capable of inducing neutralizing antibodies (*Figure 8*). However, these serological data were obtained from mice that had been injected four times, each time with 10 μg of peptides. On a molar basis, this is several hundred times more protein than is required to elicit similar neutralizing antibody levels with native virions.

IMMUNIZING ACTIVITY OF OLIGOPEPTIDES

On the basis of the deduced amino acid sequence of three FMDV strains ($A_{10}61$, $A_{12}119$ and O_1K) and data from the polypeptide fragmentation experiments

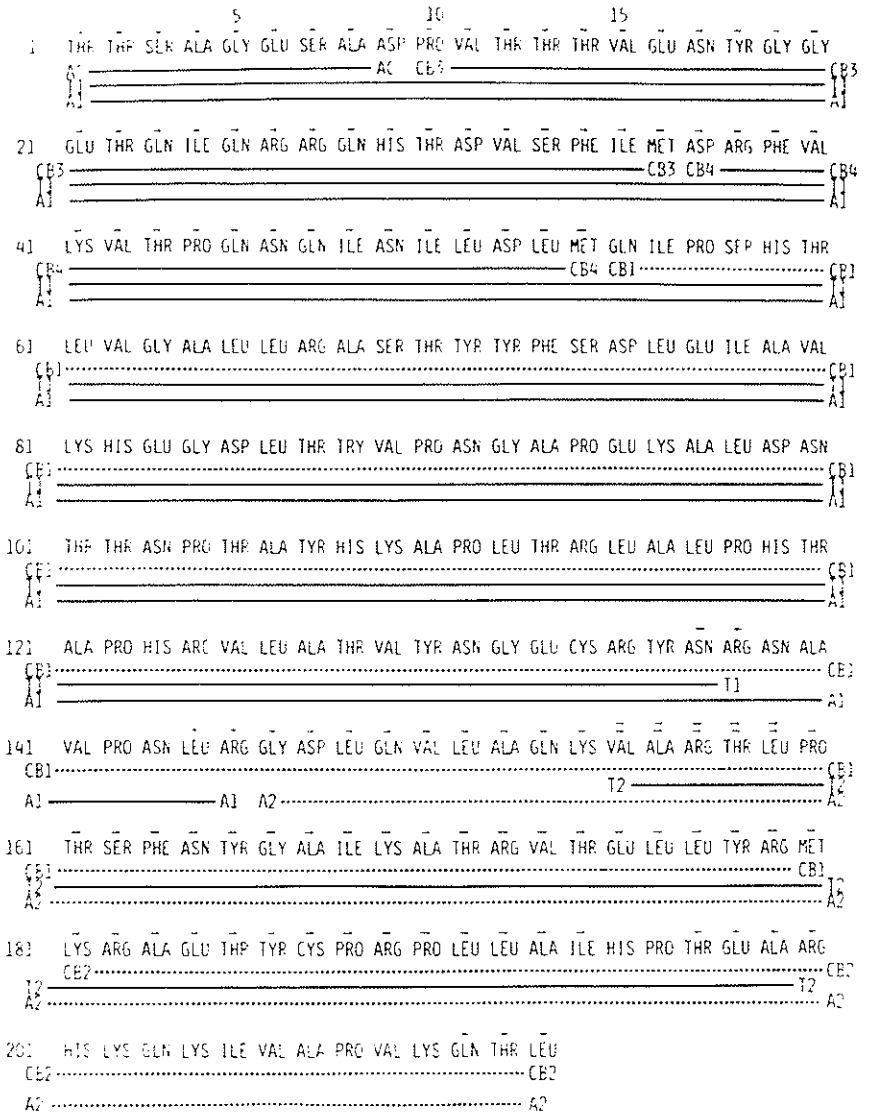

Figure 8. Immunizing activities of O_1K peptide fragments generated by cyanogen bromide or peptidases. CB = cyanogen bromide; T = trypsin; A = mouse submaxillary gland protease; ·····, neutralizing antibody-producing peptides; ———, non-inducing peptides; → → ← ←, estimated by protein sequencing. (Courtesy of Strohmaier, Franze and Adam, 1982).

(Strohmaier, Franze and Adam, 1982), Bittle *et al.* (1982) chemically synthesized three oligopeptides (i.e. Thr^{1}–Lys^{41}, Val^{141}–Pro^{160} and Arg^{201}–Leu^{214}) of O_1K, the latter two of which correspond to the highly variable regions of VP1. These were coupled separately to keyhole limpet haemocyanin (KLH) and were tested for immunogenicity by injection (four injections of 200 μg peptide) into rabbits. Virus neutralizing activity was detected only in sera of animals immunized with the Val^{141}–Pro^{160} and Arg^{201}–Leu^{214} peptides coupled to the KLH carrier (*Table 4*). Subsequently, a single inoculation of 200 μg of peptide Val^{141}–Pro^{160} was shown to protect guinea pigs from homologous virus challenge. These data suggest that the oligopeptide–KLH molecules mimic, at least in part, the configuration of the VP1 epitopes on the virus. Low levels (1%) of cross-neutralization activity against serotype C (C_3Indaial) and serotype A ($A_{10}61$) viruses were detected.

Table 4. Antibody response of rabbits to different peptides of VP1 of FMDV, type O, strain Kaufbeuren. (Courtesy of Bittle *et al.*, 1982).

Rabbit no.	Peptide	Anti-peptide antibody titre	Neutralization index (log_{10})
1	9–24	80–160	≦0·3
2	9–24	40–80	≦0·3
3	17–32	80–160	≦0·5
4	17–32	20–40	≦0·9
5	25–41	40–80	≦0·5
6	25–41	640–1280	≦0·9
7	1–41	320–640	0·9
8	1–41	320–640	0·7
9	141–160	320–640	≦3·9
10	141–160	320–640	≦3·7
11	151–160	80–160	2·9
12	151–160	160–320	1·1
13	200–213	>1280	3·5
14	200–213	160–320	3·1

An alternative approach for defining the immunogenic epitope of O_1K virus was attempted by Pfaff *et al.* (1982). They used various available methods of predicting the regions of α-helical structure in proteins to locate possible immunogenic determinants along the deduced O_1K VP1 sequence. Combining this information with the experimental data of Strohmaier, Franze and Adam (1982), they chose three regions (Leu^{144}–Leu^{159}, Ala^{167}–Lys^{181}, and Lys^{205}–Leu^{214}) for testing. Again, synthetic peptides covalently linked to the KLH carrier were used to immunize rabbits. After three inoculations, all three peptides elicited antibodies to the corresponding peptide but only Leu^{144}–Leu^{159} induced a high titre of antibody that neutralized virus when assayed in suckling mice (*Table 5*). Again, a low but measurable level of neutralizing activity against serotype C_1Obb virus (1%) was seen, although the serotype A_5WW virus was not affected. Furthermore the peptide Leu^{144}–Leu^{159} can absorb out 99% of the anti-VP1 antibody in serum raised with killed virus, suggesting that Leu^{144}–Leu^{159} is the dominant immunizing epitope of VP1.

Support for the idea that the region Asn^{134}–Arg^{154} of strain $A_{12}119$ is actually present on the surface of the virion was provided by Robertson *et al.* (1983).

Table 5. Reactivity of antipeptide antisera with the antigens, with coat protein VP1, and with FMDV serotypes O_1K, C_1O, and A_5. VP1–A = 144–159; VP1–B = 167–181; VP1–C = 206–214; VP1–A_1 = 144–151 and VP1–A_2 = 152–159. (Courtesy of Pfaff *et al.*, 1982).

		Titre in the ELISA*					
	Antigen:	Peptide		VP1	FMDV serotype		
Serum or IgG	Antiserum specificity	Free	Conjugate	O_1K FMDV	O_1K	C_1O	A_5
1	VP1–A/KLH†	3·2	3·5	3·3	3·8	1·8	NEG
2	VP1–B/KLH†	2·8	3·1	1·0	1·0	NEG	NEG
3	VP1–A/KLH†	3·6	4·4	4·4	5·1	2·3	NEG
	VP1–B/KLH†	3·3	4·0				
3A IgG§	VP1–A/KLH	3·5		3·9			
	[VP1–B/KLH]	[1·7]					
3B IgG#	[VP1–A/KLH]	[1·5]		1·2			
	VP1–B/KLH	3·1					
4	VP1–A/KLH‡	2·8		4·2	4·9	2·2	NEG
	VP1–B/KLH‡	2·3					
5	VP1–A_1/KLH†	2·0	2·2	1·7	2·2	NEG	NEG
	VP1–A_2/KLH†	2·3	2·7				
	VP1–C/KLH†	2·7	3·0				
6	VP1–A_1/KLH‡	1·0	2·5	NEG			
7	VP1–A_2/KLH‡	1·1	2·2	NEG			
8	VP1–C/KLH‡	1·3	2·7	1·9			

* $-\log_{10}$ values; NEG: values identical to those of pre-immune sera (extinction value $\varepsilon > 0{\cdot}05$ at a 1 : 10 dilution). The values shown in this table were obtained after the third injection.
† Coupling of the peptides to the carrier protein using glutaraldehyde.
‡ Coupling of the peptides to the carrier protein using *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide.
§ IgG purified by an affinity chromatography on a Sepharose–VP1–B column.
# IgG purified by an affinity chromatography on a Sepharose–VP1–A column.

These investigators demonstrated that Tyr^{136} of VP1 is preferentially iodinated *in situ*, whether native, trypsinized or bacterial-protease-cleaved viral particles are used.

Experiments with chemically synthesized oligopeptides including positions 141–160 of $A_{10}61$ and $A_{12}119$ viruses have also been reported (Clarke *et al.*, 1983). Neutralizing antibodies against the native virion can be elicited in guinea pigs immunized with these oligopeptides. In fact, the neutralizing antibodies elicited by the oligopeptides exhibited subtype specificity. Anti-$A_{10}61$ peptide (position 141–160) serum does not neutralize the $A_{12}119$ virus or vice versa. Thus, subtype specificities of serotype A viruses are likely to be associated with the 141–160 variable region.

Although the major immunogenic domains of O_1K (144–159) (Bittle *et al.*, 1982; Pfaff *et al.*, 1982) and of $A_{10}61$ or $A_{12}119$ (141–160) (Clarke *et al.*, 1983) are located within the 134–159 variable region (Cheung *et al.*, 1983), there is a VP1 immunogenic domain present on $A_{12}119$ which is absent from O_1K. VP1 fragments of O_1K (Thr^1–Arg^{138} or Thr^1–Arg^{145}) do not (Strohmaier, Franze and Adam, 1982), whereas analogous fragments of $A_{12}119$ (Thr^1–Ala^{138} or Thr^1–Arg^{145}) do (Robertson *et al.*, 1983), elicit neutralizing antibodies.

In all the above experiments, sera raised in either rabbits or guinea pigs were studied. Even though guinea pigs are used as test animals for FMDV vaccines, results obtained with these animals do not always correlate with those obtained with cattle. Thus, the efficacy of any vaccine has to be evaluated in the field.

Some future research areas

HETEROTYPIC ANTIGENIC ACTIVITIES

Traditionally, FMDV vaccine development emphasizes the importance of antigen specificity, because animals immunized against a different serotype or subtype are not protected when challenged with heterologous viruses. Recently, several groups of investigators have reported that low but clearly detectable levels of heterologous neutralizing activity could be demonstrated in sera of animals immunized with the FMDV 12S particles or with chemically synthesized peptides (Meloen and Briare, 1980; Cartwright, Chapman and Brown, 1980; Bittle *et al.*, 1982; Cartwright, Chapman and Sharpe, 1982; Cartwright, Morrell and Brown, 1982; Pfaff *et al.*, 1982). This phenomenon may be helpful in the formulation of multivalent FMDV vaccines.

The 12S particle has two kinds of activities when used to elicit neutralizing antibodies: on the one hand, it is an ineffective primary immunogen for eliciting neutralizing antibodies, although neutralizing antibody titre does increase upon multiple inoculations; on the other hand, it is effective in stimulating homotypic or heterotypic neutralizing antibodies in animals primed with killed virus of the same or different serotype (Cartwright, Morrell and Brown, 1982).

ADJUVANTS

The most important factor in the effectiveness of a vaccine is the presentation

of the desired antigenic determinants in the 'correct' conformation to the host immune system. Confusing and, at times, contradictory results have been obtained by various groups of investigators using the same immunogen. Some of this can be attributed to differences in the methods of preparing the antigens involved. Bachrach *et al.* (1982) demonstrated that $A_{12}119$ VP1 purified by three different conventional methods showed varying ability to elicit neutralizing antibodies in guinea pigs, cattle and swine. In particular, the use of lyophilized $A_{12}119$ VP1 eluted from SDS–PAGE gave a substantially lower neutralizing antibodies titre and imparted less protection to animals than did the same VP1 polypeptide without lyophilization or after DEAE-chromatography.

Vaccines are usually prepared by mixing the desired antigens with an adjuvant which helps to enhance the humoral and/or cell-mediated immune response. The stimulating effect of the adjuvant may or may not be specific in enhancing the immunogenicity of the antigen. However, regardless of whether the immune stimulation is specific or not, the antigens present in the vaccine preparation must be in the native conformation. Without exception, different adjuvants show varying degrees of enhancement for immunization with FMDV vaccines. At present, aluminum hydroxide is used as the adjuvant in FMDV vaccines. With the addition of saponin, the immune response in cattle is enhanced both after primary and after secondary vaccinations (Frenkel *et al.*, 1982). In another study, FMDV vaccines mixed with oil adjuvant (emulsion of water in mineral oil) gave longer-lasting immunity in cattle and enhanced the immunity in swine (Olascoaga *et al.*, 1982) than the aluminum hydroxide–saponin vaccine. Synthetic compounds are also being tested as adjuvants. Knudsen (1982) demonstrated that the synthetic lipid amine (CP20961) increases the antibody levels and enhances protection against FMDV in guinea pigs. Other immune stimulants, e.g. muramyl dipeptide and its derivatives or liposomes, should also be examined.

In general, the serum levels of FMDV neutralizing antibodies correlate well with the degree of protection against a particular serotype or subtype. The role of cellular immunity in protection from FMD has not been fully investigated. In two series of experiments using peptide fragments of $A_{12}119$ VP1, Bachrach *et al.* (1982) showed that both cattle and swine can be adequately protected against FMDV in the absence of high levels of neutralizing antibodies; presumably the protection is imparted by the host cellular immune response. Research work involving selective stimulation of the humoral and cellular immune responses would be extremely valuable in the development of other vaccines as well as that of FMDV vaccines.

CARRIERS

High-molecular-weight carriers are usually helpful in potentiating the immune response to low-molecular-weight antigens. Bittle *et al.* (1982), Pfaff *et al.* (1982) and Clarke *et al.* (1983) successfully immunized animals with chemically synthesized oligopeptides coupled to KLH. Of the carriers tested with the synthetic oligopeptides (bovine serum albumin, KLH, tetanus toxoid and thyroglobulin), KLH gave the best results. Unfortunately, KLH is quite expensive, and the chance of it being used in vaccine preparations is small. Synthetic carriers

designed to stimulate the host immune systems or to enhance the immunogenicity of the antigens should be examined and applied to facilitate work in this challenging task of producing an absolutely safe and more stable FMDV vaccine.

HYBRID VIRUSES

Smith, Mackett and Moss (1983) showed that a hybrid virus, in which the gene for hepatitis B surface antigen had been inserted into the vaccinia virus genome, could raise high levels of hepatitis B neutralizing antibodies in test animals. The hepatitis antigen is expressed during the replicative cycle of the vaccinia virus and released into the bloodstream of the host animal. This method of applying an antigen, as a component of a modified live vaccine, would appear to have the potential ability to impart a longer-lasting immunity than could a killed vaccine and avoids purification of the antigen. The same approach could be applied to the development of a hybrid virus vaccine for FMD.

Summary

Major contributions towards the development of an absolutely safe FMDV vaccine are evident. With the identification of VP1 as the immunogenic protein, it is possible to manufacture a subunit vaccine via biotechnology. DNA sequences encoding the VP1 protein can be introduced into a bacterium with ease; under the appropriate conditions, large amounts of VP1 can be produced in a short time. The accumulation of amino acid sequences generated by recombinant DNA techniques allows identification of antigenic domains, which are the basis of variability among serotype and subtype viruses. As a result, vaccine production by chemical synthesis of short peptides corresponding to the antigenic determinants is greatly facilitated. At present, results from experimental vaccines employing genetically engineered or chemically synthesized VP1 antigens against homologous virus infection are encouraging.

The current approach of preparing vaccine is to utilize the antigenic specificity of the virus. Since FMDV undergoes antigenic drift, variants not neutralized by type-specific serum will arise. An alternative approach is to prepare vaccines based on antigenic sites shared among all serotype and subtype viruses.

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References

ADAM, K.H. AND STROHMAIER, K. (1974). Isolation of the coat proteins of foot-and-mouth disease virus and analysis of the composition and N-terminal end groups. *Biochemical and Biophysical Research Communications* **61**, 185–192.

BACHRACH, H.L. (1977). Foot-and-mouth disease virus: properties, molecular biology

and immunology. In *Virology in Agriculture* (J.A. Romberger, Ed.), pp. 3–32. Allanheld, Osmun, Montclair.

BACHRACH, H.L. AND MCKERCHER, P.D. (1972) Immunology of foot-and-mouth disease in swine-experimental inactivated virus vaccines. *Journal of the American Veterinary Research Association* **160,** 521–526.

BACHRACH, H.L., MORGAN, D.O. AND MOORE, D.M. (1979). Foot-and-mouth disease virus immunogenic capsid protein VPT: N-terminal sequences and immunogenic peptides obtained by CNBr and tryptic cleavages. *Intervirology* **12,** 65–72.

BACHRACH, H.L., SWANEY, J.B. AND VANDE WOUDE, G.F. (1973). Isolation of the structural polypeptides of foot-and-mouth disease virus and analysis of their C-terminal sequences. *Virology* **52,** 520–528.

BACHRACH, H.L., MOORE, D.M., MCKERCHER, P.D. AND POLATNICK, J. (1975). Immune and antibody responses to an isolated capsid protein of foot-and-mouth disease virus. *Journal of Immunology* **115,** 1636–1641.

BACHRACH, H.L., MOORE, D.M., MCKERCHER, P.D. AND POLATNICK, J. (1977). An experimental subunit vaccine for foot-and-mouth disease. In *Proceedings, International Symposium on Foot-and-Mouth Disease (II), Lyon 1976.* (C. Mackowiak and R.H. Regamey, Eds). *Symposia Series in Developments in Biological Standardization,* volume 35, pp. 150–160. S. Karger, Basel.

BACHRACH, H.L., MORGAN, D.O., MCKERCHER, P.D., MOORE, D.M. AND ROBERTSTON, B.H. (1982). Foot-and-mouth disease virus: immunogenicity and structure of fragments derived from capsid protein VP3 and of virus containing cleaved VP3. *Veterinary Microbiology* **7,** 85–96.

BAHNEMANN, H.G. (1975). Binary ethylenimine as an inactivant for foot-and-mouth disease virus and its application for vaccine prodution. *Archives of Virology* **47,** 47–56.

BAHNEMANN, H.G. AUGE DE MELLO, P., ABARACON, D. AND GOMES, I. (1974). Immunogenicity in cattle of foot-and-mouth disease vaccines inactivated with binary ethylenimine. *Bulletin de l'Office International d'Epizooties, Paris* **81,** 1335–1343.

BARTELING, S.M., MELOEN, R.H., WAGENAAR, F. AND GIELKINS, A.L.J. (1979). Isolation and characterization of trypsin-resistant O_1 variants of foot-and-mouth disease virus. *Journal of General Virology* **43,** 383–393.

BAXT, B. AND BACHRACH, H.L. (1982). The adsorption and degradation of foot-and-mouth disease virus by isolated BHK-21 cell plasma membranes. *Virology* **116,** 391–405.

BECK, E., FEIL, G. AND STROHMAIER, K. (1983). The molecular basis of the antigenic variation of foot-and-mouth disease virus. *EMBO Journal* **2,** 555–559.

BITTLE, J.L., HOUGHTON, R.A., ALEXANDER, H., SHINNICK, T.M., SUTCLIFFE, J.G., LERNER, R.A., ROWLANDS, D.J. AND BROWN, F. (1982). Protection against FMDV by immunization with a chemically synthesized peptide predicted from the viral nucleotide sequence. *Nature* **298,** 30–34.

BOOTHROYD, J.C., HARRIS, T.J.R., ROWLANDS, D.J. AND LOWE, P.A., (1982). The nucleotide sequence of cDNA coding for the structural protein of FMDV. *Gene* **17,** 153–161.

BOOTHROYD, J.C., HIGHFIELD, P.E., CROSS, G.A.M., ROWLANDS, D.C., LOWE, P.A., BROWN, F. AND HARRIS, T.J.R. (1981). Molecular cloning of foot-and-mouth disease virus genome and the nucleotide sequences in the structural protein genes. *Nature* **290,** 800–802.

BROWN, F. AND SMALE, C.J. (1970). Demonstration of three specific sites on the surface of foot-and-mouth disease virus by antibody complexing. *Journal of General Virology* **7,** 115–127.

BROWN, F., CARTWRIGHT, B. AND STEWART, D.L. (1963). The effect of various inactivating agents on the viral and ribonucleic acid infectivities of foot-and-mouth disease virus and on its attachment to susceptible cells. *Journal of General Microbiology* **31,** 179–186.

BROWN, F., HYSLOP, N.St.G., CRICK, J. AND MORROW, A.W. (1963). The use of acetylethylenimine in the production of inactivated foot-and-mouth disease vaccines. *Journal of Hygiene* **61,** 337–344.

BROWN, F., NEWMAN, J., STOTT, J., PORTER, A., FRISBY, D., NEWTON, C., CAREY, N. AND FELLNER, P. (1974). Poly(C) in animal viral RNAs. *Nature* **251,** 342–344.

BURROUGHS, J.N., ROWLANDS, D.J., SANGAR, D.V., TALBOT, P. AND BROWN, F. (1971). Further evidence for multiple proteins in the foot-and-mouth disease virus particle. *Journal of General Virology* **13,** 73–84.

CAPSTICK, P.B., GARLAND, A.J., CHAPMAN, W.G. AND MASTERS, R.C. (1965). Production of foot-and-mouth disease virus antigen from BHK21 clone 13 cells grown and infected in deep suspension cultures. *Nature* **205,** 1135.

CARTWRIGHT, B., CHAPMAN, W.G. AND BROWN, F. (1980). Serological and immunological relationships between the 140S and 12S particles of FMDV. *Journal of General Virology* **50,** 369–375.

CARTWRIGHT, B., CHAPMAN, W.G. AND SHARPE, R.T. (1982). The stimulation by heterotypic antigens of foot-and-mouth disease virus antibodies in vaccinated cattle. *Research in Veterinary Science* **32,** 338–342.

CARTWRIGHT, B., MORRELL, D.J. AND BROWN, F. (1982). Nature of the antibody response to the foot-and-mouth disease virus particle, its 12S protein subunit and the isolated immunizing polypeptide VP1. *Journal of General Virology* **63,** 375–381.

CAVANAGH, D., SANGAR, D.V., ROWLANDS, D.F. AND BROWN, F. (1977). Immunogenic and cell attachment sites of FMDV: Further evidence for their location in a single capsid polypeptide. *Journal of General Virology* **35,** 149–158.

CHEUNG, A., DELAMARTER, J., WEISS, S. AND KÜPPER, H. (1983). Comparison of the major antigenic determinants of different serotypes of foot-and-mouth disease virus. *Journal of Virology*, in press.

CLARKE, B.E., CARROLL, A.P., ROWLANDS, D.J., NICHOLSON, X., HOUGHTON, R.A., LERNER, R.A. AND BROWN, F. (1983). Synthetic peptides mimic subtype specificity of foot-and-mouth disease virus. *FEBS Letters* **157,** 261–264.

COHEN, S., CHANG, A.C.Y. AND HSU, L. (1972). Nonchromosomal antibiotic resistance in bacteria: Genetic transformation of *E. coli* by R-factor DNA. *Proceedings of the National Academy of Sciences of the United States of America* **69,** 2110–2114.

DERYNCK, R., REMAUT, E., SAMAN, E., STANSSENS, P., DE CLERCG, E., CONTENT, J. AND FIERS, W. (1980). Expression of human fibroblast interferon gene in *Escherichia coli*. *Nature* **287,** 193–197.

DOEL, T.R., SANGAR, D.V., ROWLANDS, D.J. AND BROWN, F. (1978). A reappraisal of the biochemical map of foot-and-mouth disease virus RNA. *Journal of General Virology* **41,** 395–404.

DIETZSCHOLD, B., KAADEN, O.R. AND AHL, R. (1972). Hybridization studies with subtypes and mutants of foot-and-mouth disease virus type O. *Journal of General Virology* **15,** 171–174.

FORSS, S. AND SCHALLER, H. (1982). A tandem repeat gene in a picornavirus. *Nucleic Acids Research* **10,** 6441–6450.

FRENKEL, H.S. (1951). Research on foot-and-mouth disease III. The cultivation of the virus in explanation of tongue epithelium of bovine animals. *American Journal of Veterinary Research* **12,** 187.

FRENKEL, S., BARENDREGT, L.G., KLOOSTERMAN, E.G. AND TALMAN, F.P. (1982). Serological response of calves to aluminum hydroxide gel FMD vaccine with or without saponin. Influence of genetic differences on this response. In *Proceedings of the 16th Conference of the Foot-and-Mouth Disease Commission*, pp. 211–221. Office International des Epizooties, Paris, France.

GRUBMAN, M.J. (1980). The 5′ end of foot-and-mouth disease virion RNA contains a protein covalently linked to the nucleotide pUp. *Archives of Virology* **63,** 311–315.

HARDY, K., STAHL, S. AND KÜPPER, H. (1981). Production in *B. subtilis* of hepatitis B core antigen and of major antigen of foot-and-mouth disease virus. *Nature* **293,** 481–483.

HARRIS, T.J.R. (1979). The nucleotide sequence at the 5′ end of foot-and-mouth disease virus RNA. *Nucleic Acids Research* **7,** 1765–1785.

HARRIS, T.J.R. AND BROWN, F. (1976). The location of the poly(C) tract in the RNA of foot-and-mouth disease virus. *Journal of General Virology* **33,** 493–501.

HOFSCHNEIDER, P.H., BUERGELT, E., KAUZMANN, M., MUSSGAY, M., FRANZ, R., AHL, R., BOEHM, H., STROHMAIER, K., KÜPPER, H. AND OTTO, B. (1981). Studies on the antigenicity and immunogenicity of the foot-and-mouth disease viral protein VP1 expressed in *E. coli*. In *Prospective Biological Products for Viral Diseases. 6th Munich Symposium on Microbiology* (P.A. Bachman, Ed.), pp. 105–113. Taylor and Francis, London.

ITAKURA, K., HIROSE, T., CREA, R., RIGGS, A.D., HEYNEKER, H.L., BOLIVAR, F.P. AND BOYER, H.W. (1977). Expression in *Escherichia coli* of a chemically synthesized gene for the hormone somatostatin. *Science* **198,** 1056–1063.

KAADEN, O.R., ADAM, K.H. AND STROHMAIER, K. (1977). Induction of neutralizing antibodies and immunity in vaccinated guinea pigs by cyanogen bromide peptides of VP3 of foot-and-mouth disease virus. *Journal of General Virology* **34,** 397–400.

KELLY, T.J. AND SMITH, H.O. (1970). A restriction enzyme from *Haemophilus influenzae* II. Base sequence of the recognition site. *Journal of Molecular Biology* **51,** 379–392.

KING, A.M.Q., UNDERWOOD, B.O., MCCAHON, D., NEWMAN, J.W.I AND BROWN, F. Biochemical identification of viruses causing the 1981 outbreaks of foot-and-mouth disease in the UK. *Nature* **293,** 479–480.

KLEID, D.G., YANSURA, D., SMALL, B., DOWBENKO, D., MOORE, D.M., GRUBMAN, J.J., MCKERCHER, P.D., MORGAN, D.O., ROBERTSON, B.H. AND BACHRACH, M.L. (1981). Cloned viral protein vaccine for foot-and-mouth disease: responses in cattle and swine. *Science* **214,** 1125–1129.

KNUDSEN, R.C. (1982). Adjuvant effects of two formulations of CP20961, a synthetic lipid amine, for FMD virus vaccine in guinea pigs. In *Proceedings of the 16th Conference of the Foot-and-Mouth Disease Commission*, pp. 184–197. Office International des Epizooties, Paris, France.

KÜPPER, H., DELAMARTER, J., OTTO, B. AND SCHALLER, H. (1982). Expression of major foot-and-mouth disease antigen in *E. coli*. In *Proceedings of the IVth International Symposium on Genetics of Industrial Microorganisms* (Y. Ikeda and T. Beppu, Eds), pp. 222–226. Kodansha Limited, Tokyo.

KÜPPER, H., KELLER, W., KURZ, CH., FORSS, S., SCHALLER, H., FRANZE, R., STROHMAIER, K., MARQUARDT, O., ZASLAVSKY, V.G. AND HOFSCHNEIDER, H. (1981). Cloning of cDNA of the major antigen of foot-and-mouth disease virus and expression in *E. coli*. *Nature* **289,** 555–559.

KURZ, C., FORSS, S., KÜPPER, H., STROHMAIER, K. AND SCHALLER, H. (1981). Nucleotide sequence and corresponding amino acid sequence of the gene for the major antigenic sites of foot-and-mouth disease virus. *Nucleic Acids Research* **9,** 1919–1931.

LAPORTE, J. AND LENOIR, G. (1973). Structural proteins of foot-and-mouth disease virus. *Journal of General Virology* **20,** 161–168.

LAPORTE, J., GROSCLAUDE, J., WANTYGHEM, J., BERNARD, S. AND ROUZE, P. (1973). Neutralisation en culture cellulaire du pouvoir infectieux du virus de la fièvre aphteuse par des serums provenant de porcs immunisés à l'aide d'une protéine virale purifiée. *Comptes rendus hebdomadaires des séances de l'Académie des sciences. Series D* **276,** 3399–3402.

LA TORRE, J.L., GRUBMAN, M.J., BAXT, B. AND BACHRACH, H.L. (1980). Structural polypeptides of aphtovirus are phosphoproteins. *Proceedings of the National Academy of Sciences of the United States of America* **77,** 7444–7447.

MCKERCHER, P.D. AND FARRIS, H.E. (1967) Foot-and-mouth disease in swine: Response to inactivated vaccines. *Archiv für die gesamte Virusforschung* **22,** 451–461.

MAKOFF, A.J., PAYNTER, C.A., ROWLANDS, D.J. AND BOOTHROYD, J.C. (1982). Comparison of the amino acid sequence of the major immunogen from three serotypes of foot-and-mouth disease virus. *Nucleic Acids Research* **10,** 8285–8295.

MAXAM, A. AND GILBERT, W. (1977). A new method for sequencing DNA. *Proceedings of the National Academy of Sciences of the United States of America* **74,** 560–564.

MATHEKA, H.D. AND BACHRACH, H.L. (1975). N-Terminal amino acid sequences in the major capsid proteins of foot-and-mouth disease virus types A, O and C. *Journal of Virology* **16,** 1248–1253.

MELOEN, R.H. (1976). Localisation on foot-and-mouth disease virus (FMDV) of an antigenic deficiency induced by passage in BHK cells. *Archives of Virology* **51,** 299–306.

MELOEN, R.H. AND BRIAIRE, J. (1980). A study of the cross-reacting antigens on the intact foot-and-mouth disease virus and its 12S subunits with antisera against the structural proteins. *Journal of General Virology* **51,** 107–116.

MELOEN, R.H., ROWLANDS, D.J. AND BROWN, F. (1979). Comparison of the antibodies elicited by the individual structural polypeptides of foot-and-mouth disease and polio viruses. *Journal of General Virology* **45,** 761–763.

MELOEN, R.H., BRIARE, J., WOORTMEYER, R.J. AND VAN ZAANE, D. (1983). The main antigenic determinant detected by neutralizing monoclonal antibodies on the intact foot-and-mouth disease virus particle is absent from isolated VP1. *Journal of General Virology* **64,** 1193–1198.

MOORE, D.M. (1983). Introduction of a vaccine for foot-and-mouth disease through gene cloning. *Beltsville Symposia in agricultural research, Genetic engineering in agriculture, Recombinant Technology, May 1982*, pp. 132–142. Allanheld, Osmun, Montclair.

MOORE, D.M. AND COWAN, K.M. (1978). Effect of trypsin and chymotrypsin on the polypeptides of large and small plague variants of foot-and-mouth disease virus: relationship to specific antigenicity and infectivity. *Journal of General Virology* **41,** 549–567.

MORGAN, D.O., MCKERCHER, P.D. AND BACHRACH, H.L. (1970). Quantitation of the antigenicity and immunogenicity of purified foot-and-mouth disease virus vaccine for swine and steers. *Applied Microbiology* **20,** 770–774.

MOWAT, G.N. AND CHAPMAN, W.G. (1962). Growth of foot-and-mouth disease virus in fibroblastic cell line derived from hamster kidneys. *Nature* **194,** 253–255.

MOWAT, G.N., BARR, D.A. AND BENNETT, J.H. (1969). The development of an attenuated foot-and-mouth disease virus vaccine by modification and cloning in tissue cultures of BHK21 cells. *Archiv für die gesamte Virusforschung* **26,** 341–354.

OLASCOAGA, P.C., SUTMOELLER, P., FERNANDEZ, A. AND ABARACON, D. (1982). FMD virus production and control of vaccines in South America. In *Proceedings of the 16th Conference of the Foot-and-Mouth Disease Commission*, pp. 139–152. Office International des Epizooties, Paris, France.

PEREIRA, H. (1977). Subtyping of foot-and-mouth disease virus. In *Proceedings, International Symposium on Foot-and-Mouth Disease (II) Lyon 1976* (C. Mackowiak and R.H. Regamey, Eds). *Symposia Series in Developments in Biological Standardization*, volume 35, pp. 167–174. S. Karger, Basel.

PFAFF, E., MUSSGAY, M., BOEHM, H.O., SCHULZ, G.E. AND SCHALLER, H. (1982). Antibodies against a preselected peptide recognize and neutralize foot-and-mouth disease. *EMBO Journal* **1,** 869–874.

REMAUT, E., STANSSENS, P. AND FIERS, W. (1981). Plasmid vectors for high-efficiency expression controlled by the pL promoter of coliphage lambda. *Gene* **15,** 81–88.

ROBERTSON, B.H., MOORE, D.M., GRUBMAN, M.J. AND KLEID, D.G. (1983). Identification of an exposed region of the immunogenic capsid polypeptide VP1 on foot-and-mouth disease virus. *Journal of Virology* **46,** 311–316.

ROWLANDS, D.J., HARRIS, T.J.R. AND BROWN, F. (1978). More precise location of the polycytidylic acid tract in foot-and-mouth disease virus RNA. *Journal of Virology* **26,** 335–343.

ROWLANDS, D.J., SANGAR, D.V. AND BROWN, F. (1971). Relationship of the antigenic structure of foot-and-mouth disease virus to the process of infection. *Journal of General Virology* **13,** 85–93.

SANGAR, D.V. (1979). The replication of picornaviruses. *Journal of General Virology* **45,** 1–13.

SANGER, F., NICKLEN, S. AND COULSON, A.R. (1977). DNA sequencing with chain-termination inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* **74,** 5463–5467.

SANGAR, D.V., BLACK, D.N., ROWLANDS, D.J. AND BROWN, F. (1977). Biochemical mapping

of the foot-and-mouth disease virus genome. *Journal of General Virology* **35,** 281–297.

SANGAR, D.V., ROWLANDS D.J., HARRIS, T.J.R. AND BROWN, F. (1977). Protein covalently linked to foot-and-mouth disease virus RNA. *Nature* **268,** 648–650.

SANGAR, D.V., ROWLANDS, D.J., SMALE, C.J. AND BROWN, F. (1973). Reaction of glutaraldehyde with foot-and-mouth disease virus. *Journal of General Virology* **21,** 399–406.

SANGAR, D.V., BLACK, D.M., ROWLANDS, D.J., HARRIS, T.J.R. AND BROWN, F. (1980). Location of initiation site for protein synthesis on foot-and-mouth disease virus RNA by in vitro translation of defined fragments of the RNA. *Journal of Virology* **33,** 59–68.

SCODELLER, E.A., LEBENDIKER, M.A., DUBRA, M.S., BASARAB, O., LA TORRE, J.L. AND VASQUEZ, C. (1982). Inactivation of FMD virus by activation of virion-associated endonuclease. In *Proceedings of the 16th Conference of the Foot-and-Mouth Disease Commission*, pp. 43–50. Office International des Epizooties, Paris, France.

SKINNER, H.H. (1952). One-week-old white mice as test animals in foot-and-mouth disease research. In *Proceedings of the 15th International Veterinary Congress, Stockholm 1952*, Part 1, pp. 195–199.

SKINNER, H.H. (1959). The immunogenicity of strains of the virus of foot-and-mouth disease modified by serial passage in white mice and chick embryos. In *Proceedings, 16th International Veterinary Congress, Madrid*, pp. 391–393.

SKINNER, H.H. (1960). Some techniques for producing and studying attenuated strains of the virus of foot-and-mouth disease. *Bulletin de l'Office International d'Epizooties, Paris* **53,** 634–650.

SMITH, G.L., MACKETT, M. AND MOSS, B. (1983). Infectious vaccinia virus recombinants that express hepatitis B virus surface antigen. *Nature* **302,** 490–495.

SMITH, H.O. AND WILCOX, K.W. (1970). A restriction enzyme from *Haemophilus influenzae* I. Purification and general properties. *Journal of Molecular Biology* **51,** 379–391.

STROHMAIER, K. AND ADAM, K.H. (1974). Comparative electrophoretic studies of foot-and-mouth disease virus proteins. *Journal of General Virology* **22,** 105–114.

STROHMAIER, K., FRANZE, R. AND ADAM, K.H. (1982). Location and characterization of the antigenic portion of the FMDV immunizing protein. *Journal of General Virology* **59,** 295–306.

STROHMAIER, K., WITTMANN-LIEBOLD, B. AND GEISSLER, A.-W. (1978). The N-terminal sequence of three coat proteins of foot-and-mouth disease virus. *Biochemical and Biophysical Research Communications* **85,** 1640–1645.

TALBOT, P., ROWLANDS, D.J., BURROUGHS, J.N., SANGAR, D.V. AND BROWN, F. (1973). Evidence for a group protein in foot-and-mouth disease virus particles. *Journal of General Virology* **19,** 369–380.

VANDE WOUDE, G.F., SWANEY, J.B. AND BACHRACH, H. (1972). Chemical and physical properties of foot-and-mouth disease virus: a comparison with Maus Elberfeld virus. *Biochemical and Biophysical Research Communications* **48,** 1222–1229.

VASQUEZ, C., DENOYA, C.D., LA TORRE, J.L. AND PALMA, E.L. (1979). Structure of foot-and-mouth disease virus capsid. *Virology* **97,** 195–200.

VILLA-KOMAROFF, L., EFSTRATIADIS, A., BROOME, S., LOMEDICO, P., TIZARD, R., NABER, S.P., CHICK, W.L. AND GILBERT, W. (1978). A bacterial clone synthesising proinsulin. *Proceedings of the National Academy of Sciences of the United States of America* **75,** 3727–3731.

WESTERGAARD, J.M. (1982). The epidemiology of FMD outbreaks on the Islands of Funen and Zeeland in Denmark. In *Proceedings of the 16th Conference of the Foot-and-Mouth Disease Commission*, pp. 527–550. Office International des Epizooties, Paris, France.

WILD, T.F. AND BROWN, F. (1967). Nature of the inactivating action of trypsin on foot-and-mouth disease virus. *Journal of General Virology* **1,** 247–250.

WILD, T.F., BURROUGHS, J.N. AND BROWN, F. (1969). Surface structure of foot-and-mouth disease virus. *Journal of General Virology* **4,** 313–320.

WILLIAMS, D.C., VAN FRANK, R.M., MUTH, W.L. AND BENNET, J.P. (1982). Cytoplasmic inclusion bodies in *Escherichia coli* producing biosynthetic human insulin proteins. *Science* **215**, 687–689.